THE NEUTROPHIL IN ACUTE MYOCARDIAL INFARCTION

by

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To Mum and Dad

DECLARATION

The work described in this thesis was carried out by the author as part of a research group and is the work of the author with the exception of the following:

Clinical assessment and medical supervision of the patients was carried out by Dr D Bell, Lecturer in Medicine, University Department of Medicine, Royal Infirmary of Edinburgh.

Determination of diene conjugation products in plasma samples was performed by Mr R Dawkes, Senior MLSO, University Department of Medicine, Royal Infirmary of Edinburgh.

Operation of the gamma camera was performed by Ms F Taddei, Senior Chief Physiological Measurement Technician and Ms S Turnbull, Senior Radiographer.

Technical support and software was provided for the gamma camera by Dr JJ Nicoll, Senior Physicist, Department of Medical Physics and Medical Engineering.

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ETHICAL CONSIDERATIONS

Studies involving patients were approved by the Medical Sub-Committee of the Lothian Ethical Committee.

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* Former name

ABBREVIATIONS

ANT	anterior
AMI	acute myocardial infarction
APSAC	anisoylated plasminogen streptokinase activator complex
BP	British pharmacopoeia
Br^-	bromide anion
CCU	coronary care unit
Cl^-	chloride anion
CK	creatine kinase
COAD	chronic obstructive airways disease
CGD	chronic granulomatous disease
CGL	circulating granulocyte pool
^{51}Cr	chromiun-51
DNA	deoxyribonucleic acid
ECG	electrocardiogram
EDTA	ethylenediaminetetraacetic acid
ELAM-1	endothelial-leucocyte adhesion molecule
ELISA	enzyme-linked immunoassay
ESR	erythrocyte sedimentation rate
ESR	electron spin resonance spectroscopy
F	female
FMLP	formyl-methionyl-leucyl-phenylalanine
^{67}Ga	gallium-67
H_2O	water
H_2O_2	hydrogen peroxide
Hb	haemoglobin
HOCl	hypochlorous acid
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid

HETES	hydroxytetranoic acid
HRPO	horseradish peroxidase
HSA	human serum albumin
I^-	iodide anion
^{125}I	iodine-125
ICAM-1	inter-cellular adhesion molecule
IgG	immunoglobulin G
IHD	ischaemic heart disease
IL-1	interleukin 1
INF	inferior
^{111}In	indium-111
kD	kilo Dalton
LAD	leucocyte adhesion deficiency
LAO	left anterior oblique
LDH	lactate dehydrogenase
LF	lactoferrin
LFA-1	lymphocyte functional antigen
LPS	lipopolysaccharide
LRPRP	leucocyte rich platelet rich plasma
LT	lymphotoxin
LTB_4	leukotriene B_4
LV	left ventricle
LVEF	left ventricular ejection fraction
M	male
MCV	mean cell volume
MGP	marginal granulocyte pool
MPO	myeloperoxidase
M-PRM	mono-poly resolving medium

MUGA	multiple gated acquisition method
N	normal
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
O_2	oxygen
O_2^-	superoxide anion
$^{\cdot}OH$	hydroxyl radical
PBS	phosphate buffered saline
PGI_2	prostacyclin
PL-9,11-LA'	phospholipid 9,11 linoleic acid
PL-9,12-LA	linoleic acid
PMA	phorbol myristate acetate
PNE	plasma neutrophil elastase
PO_4	phosphate
PPP	platelet poor plasma
PVP	polyvinylpyrrolidone
PYP	pyrophosphate
RAO	right anterior oblique
RBC	red blood cell
RIA	radioimmunoassay
RNA	ribonucleic acid
ROI	region of interest
RT	room temperature
RV	right ventricle
SCN^-	thiocyanate anion
SD	standard deviation
SEM	standard error of the mean
SOD	superoxide dismutase

SPET	single photon emission computed tomography
SPSS	statistical package for social sciences
TBA	thiobarbituric acid test
TBGP	total blood granulocyte pool
TNF	tumour necrosis factor
TRIS	2-amino-2-(hydroxymethyl)propane-1,3-diol
VLA	very late appearing antigens
WBC	white blood cell count

ABSTRACT

Death of heart muscle cells, manifested as acute myocardial infarction, is a major cause of mortality in the western world. Although coronary artery occlusion is the principal cause, animal studies have suggested that infiltration of neutrophils as part of the inflammatory response to ischaemic injury, may be significant in the extension of tissue damage.

As a hypothesis I suggest that neutrophil activation may also play a significant role in extending tissue injury in man and my thesis examines the role of the neutrophil in the clinical syndrome of myocardial infarction.

Firstly, methods for isolating and radiolabelling neutrophils were developed. These, along with measurement of established markers of neutrophil activation and free radical activity, were used to assess neutrophil involvement in myocardial infarction in man.

The single-step isolation procedure developed provided a simple and easy means of isolating an essentially "pure" preparation of cells with a minimum of "handling". That this method resulted in isolation of a viable cell population was evidenced by normal kinetics and uptake into sites of infection and inflammation in vivo.

Using this labelling method it was shown that there is uptake of autologous 111-Indium labelled neutrophils in the heart, in patients with recent myocardial infarction. The time interval from onset of chest pain to injection of labelled cells was the only factor shown to determine the outcome of imaging and suggests that the stimulus for cell infiltration may be early and transient.

Detection of increased neutrophil elastase by radioimmunoassay and the non-peroxide diene conjugated isomer of linoleic acid by high performance liquid chromatography in the plasma of these patients demonstrated increased neutrophil activation and free radical activity in acute myocardial infarction in man.

Coronary reperfusion, effected by intravenous thrombolysis, might be thought to be associated with increased neutrophil activation, but the results showed a reduction in the intensity of the inflammatory response as assessed by uptake of radiolabelled autologous neutrophils, abolition of the late peak of neutrophil activation and a similar degree of free radical activity between patients treated with and without thrombolysis. This is consistent with a reduction rather than an exaggeration of the inflammatory response and conflicts with current views on "reperfusion injury".

In conclusion, following myocardial infarction in man there is evidence of neutrophil infiltration, release of neutrophil elastase and increased free radical activity indicating that neutrophil activation is an early phenomenon in acute myocardial infarction. However coronary reperfusion does not appear to further potentiate this activation.

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CHAPTER 1

INTRODUCTION

Acute myocardial infarction is one of the commonest afflictions of the westernised world. It is almost invariably caused by coronary artery occlusion by thrombus, leading to infarction or death of the myocyte.

Animal studies have suggested that the infiltration of neutrophils, as part of the acute inflammatory response to ischaemic injury, may contribute significantly to the extension of tissue damage.
The aim of this thesis was to determine if the neutrophil plays a significant role in myocardial infarction in man.

Before doing so it is helpful to outline the nature of infarction.

1.1.1 ACUTE MYOCARDIAL INFARCTION

Historical Background

Although angina pectoris was recognised in the 18th century (Heberden 1772), the clinical syndrome of myocardial infarction was not known until the 20th century.

In the intervening period, Weigert, a German pathologist, described the similarities of infarcts of the myocardium to those found in other areas of the body. In his paper entitled "Ueber die pathologischen Gerinnungsvorgange", he documented the gross and macroscopic appearances of myocardial infarction, and also the healing process which resulted in the formation of scar tissue (Weigert, 1880). By 1894, Gibson and Muir described two patients in Ward 22 of the Royal Infirmary of Edinburgh who sustained "morbid changes which the muscular wall of the heart undergoes, in a consequence of local nutritive disturbances", noting this was the condition myomalacia cordis, described by Zeigler, (1880).

The clinical syndrome of myocardial infarction and associated pathogenic mechanisms were first described in the early 1900's by two groups at around the same time (Obratzsov **et al** in 1910; Herrick 1912), although the main credit in the western world is usually given to Herrick. In their paper, Obstratzsov and Stratletskov presented post mortem details of five patients with acute myocardial infarction, with coronary thrombosis evident in only three of the five. Herrick, in describing this syndrome postulated even at this early date that "hope for the damaged myocardium lies in the direction of securing a supply of blood so as to restore as far as possible its functional integrity"

Although these papers excited the medical community, the exact nature of the cause of myocardial infarction was unclear. In the 1920's, several studies revealed many instances where severe atherosclerosis was apparent but coronary thrombosis could not be detected following myocardial infarction. Particular examples of this include comments by Friedberg **et al**, (1939) on the possibility that myocardial infarction may occur in the absence of coronary artery occlusion.

Such confusion eventually led clinicians and pathologists to suggest that myocardial infarction could not only occur in the absence of coronary thrombosis, but that coronary occlusion may be the result rather than the cause of acute myocardial infarction (Baroldi **et al**, 1976).

The debate continued for several decades. Two important factors generating much of the confusion regarding the variable importance of coronary thrombosis in various types of myocardial infarction occurred because the distinction between transmural and non-transmural and the pathogenesis of sudden cardiac death had not been clarified.

It is now generally accepted that the initiating event in acute

myocardial infarction, is a critical reduction in the lumen in one or more coronary artery. The detailed and painstaking work of Davies, (1984) clearly showed the role of coronary thrombosis in the pathogenesis of acute myocardial infarction. Similar studies have been reported by Falk **et al**, (1987).

Thrombus is commonly formed on the surface of a ruptured or fissured atheromatous plaque and this thrombus produces occlusion of the coronary artery leading to downstream anoxia.

1.1.2 **Definition**

The term myocardial infarction refers to the death of a part or all of a region of myocardium. It occurs when ischaemia has been sufficiently prolonged to induce irreversible injury of the affected cells so that necrosis occurs even after the restoraton of blood flow.

Much that is known of the course of myocardial infarction and the subsequent healing process was learned from experimental animal models in which temporary coronary occlusion was followed by reperfusion (Jennings **et al**, 1960; Karsner **et al**, 1916).

In anaesthetised open-chest dogs, myocytes rendered severely ischaemic were shown to remain viable for at least 15 minutes (Jennings **et al** 1960). If perfusion is re-established at this time, then infarction may be avoided with an eventual recovery of cellular metabolism, ultrastructure and contractile function. When coronary occlusion is extended beyond this period of 15 minutes increasing numbers of myocytes become irreversibly injured and by 40 minutes much of the ischaemic subendocardial zone will be irreversibly injured (Jennings **et al**, 1960). As the duration of coronary occlusion increases

a "wavefront" of cell death will gradually progress from the subendocardium to the subepicardium. Similar patterns of cell death are seen in other species such as rabbits (Connelly **et al**, 1982), pigs (Klein **et al** 1984) and baboons (Geary **et al**, 1982).

From these and other studies it is recognised that following coronary artery occlusion, ischaemic myocytes do not die instantaneously, that mildly ischaemic myocytes may survive indefinitely and within the region that undergoes infarction, not all myocytes die simultaneously (Reimer **et al**, 1979). These concepts are crucially important and form the basis for experimental and clinical efforts to design therapy that limits infarct size (Hillis **et al**, 1977).

The events involved with healing of experimental myocardial infarcts were also characterised in canine models and correlated with histologic observations. (Karsner **et al**, 1916).

The nature of the subsequent healing associated with myocardial infarction in man was examined first by Levine who correlated the age of infarct with gross histologic findings (Levine **et al**, 1929). Although this paper proved a valuable start, insufficient cases and lack of detailed examination provided limited conclusions. Ten years later, the definitive pathological study on the process of healing after myocardial infarction was conducted by Mallory **et al** (1939) and is the basis for the present day understanding of the events following infarction.

1.1.3 **Myocardial Infarction in Man**

Mallory concluded from his studies that the speed of healing of infarcts in humans was similar in most respects to that of experimental lesions in animals except that it is slower (Mallory 1939).

In man, the site and the extent of infarction is governed by a

multitude of variables and include the site and severity of the atheroma in the arteries supplying the zone of infarction, the dynamic nature of the occluding thrombus, the presence of separate lesions in other coronary arteries, collateral circulation and myocardial oxygen demand.

Irreversibly damaged myocytes do not regenerate, so are removed and eventually replaced by scar tissue. While the ischaemic death of the myocyte is rapid, the process of repair is longer and may require up to 4 to 6 weeks. In commenting on the speed of healing after myocardial infarction Mallory concluded that it "is in part dependent upon the size and position of the infarct and in part due to the state of the remaining myocardial circulation" (Mallory **et al**, 1939).

As the endogenous degradative enzymes of myocytes are insufficient to effect complete dissolution of dead myocytes, removal of necrotic cells depends on the influx of polymorphonuclear neutrophils and macrophages as part of the acute inflammatory response to tissue injury (Bing 1971/2).

The presence of marginating polymorphonuclear neutrophils within the microvasculature and in the surrounding interstitial tissues may be observed within the first few hours of infarction. This infiltration starts peripherally, spreading centrally from the epicardium to the endocardium. The number of neutrophils present in the tissue increases with time and reaches a peak at around 3 to 4 days. At around 48 hours those neutrophils reaching the site of infarct do not recirculate and undergo degenerative changes in-situ. By the 4th to 5th day the influx begins to subside with macrophages becoming more prominent (Mallory **et al**, 1939).

The progressive removal of injured myocytes continues and is followed by ingrowth of new capillary buds and fibroblasts and within the tenth

day a distinct rim of granulation tissue is present. Between the 2nd and 4th week, 'organisation' of the infarct takes place and the process of repair is generally complete within 3 to 6 months.

1.1.4 **Harmful Effects Associated with the Acute Inflammatory Response**

The acute inflammatory response as it relates to myocardial infarction has not been widely studied. It is essential for the resolution and repair of the infarcted area but recent studies also indicate that the inflammatory response, or some facet of this response, may cause additional injury to reversibly damaged myocytes and thus may extend the ultimate size of infarct. It is therefore relevant to describe the events constituting the inflammatory response, particularly those aspects, which when poorly regulated, may potentionally lead to extension of tissue injury.

1.2 INFLAMMATION

The inflammatory response is an integral part of the host system of defence against damage and has been well characterised (Wilkinson, 1974).

It was first recognised in ancient times by the appearances it produced in the skin and other surfaces of the body. Its manifestations were described by the Roman encyclopaedist Celsus (30BC-38AD) as "Rubor et tumor cum calor et dolor - redness and swelling with heat and pain" These changes are still known as the cardinal signs of inflammation.

The first clear statement on the modern concept of inflammation was given by John Hunter (1794), who after first hand study of injured tissues concluded that "inflammation itself should not to be considered as a disease, but as a salutary operation consequent either to some violence or some disease". All modern definitions since then are restatements of this basic concept.

The earliest workers to examine living inflamed tissue were a group of British pathologists, Thomson (1813), Wharton-Jones (1842), Addison (1843) and Waller (1846), who between them described all the essential features of the early stages of inflammation. Their accounts however, were largely ignored and it was not until 1882, when Conheim provided one of the first microscopic descriptions of inflammation in injured blood vessels in thin, transparent membranes, such as the tongue and mesentery of the frog, that the basis of the cardinal signs were generally accepted. His descriptions of the initial vasodilation, changes in blood flow, the subsequent oedema due to increased vascular permeability and the characteristic leucocyte migration are still valid to modern medicine.

Today it is known that any event causing tissue injury may invoke an

inflammatory reaction, which may be considered not as a single process, but rather a number of inter-related processes.

The inflammatory process is generally thought of as an immediate reaction to injury which gives rise to an acute inflammatory response. In contrast, chronic inflammation results from injurious stimuli that are persistent, often for weeks or months, leading to a predominantly proliferative (fibroblastic) rather than exudative reaction.

Because acute inflammation associated with invasion of the tissues by pathogenic micro-organisms is so common, for a time inflammation was synonymous with infection. But it is now clear that in addition to infective agents, inflammation may be initiated by any form of tissue trauma be it mechanical, chemical, thermal and in the context of this study, ischaemic.

Following tissue injury, the host will mount a characteristic acute inflammatory response. Despite the diversity of the damaging agents and tissues involved in inflammation, the same chemical mediators are released and so the immediate inflammatory response is similar. The extent and intensity of the response is dependent on the severity of injury and reactive capability of the host.

1.2.1 **Mediators of Inflammation**

Although injury precipitates the inflammatory response, it is the subsequently released chemicals that mediate the response. These mediators, which are large in number, can originate from plasma, cells or damaged tissue.

A large number of mediators exist, however most workers accept that those most closely associated with myocardial infarction are split products of the complement system and products of arachidonic acid

metabolism. Table 1(i) details substances that mediate the inflammatory response.

Table 1(i)

Mediators of Inflammation

1. Vasoactive Amines		Histamine, Serotonin
2. Plasma Proteases	a)	the kinin system (bradykinin, kallikrein)
	b)	the complement system (C3a, C5a, C5b-C9)
	c)	the coagulation/fibrinolytic system (fibrinpeptides, fibrin degradation products)
3. Arachidonic Acid Metabolites		
	a)	via the cyclo-oxygenase pathway (endoperoxides, prostaglandins, thromboxane)
	b)	via the lipoxygenase pathway (leukotrienes, HPETE, HETE)
4. Lysosomal constituents		(neutral proteases)
5. Oxygen-derived free radicals		($O^{\cdot}_2$, $^{\cdot}OH$)
6. Cytokines		(IL-1, TNF)

The acute inflammatory response itself can be divided into three major components; the vascular response; the formation of exudate; and the cellular response.

1.2.2 Vascular Response

a)Alteration of Blood Flow

Immediately after injury, the vessels at the site of inflammation become dilated. As a consequence of the local dilation, there is increased blood flow and delivery of increased numbers of leucocytes to the tissue site. The resulting hyperaemia and subsequent stasis of the circulation produce changes in the intravascular pressure and orientation of blood cells in the local microcirculation. Both leucocyte accumulation and vascular leak were shown by Rampart and co-workers (1986) to be markedly enhanced in the rabbit by local administration of vasodilators when combined with neutrophil chemoattractants such as C5a and formyl-methionyl-leucyl-phenylalanine (FMLP).

A number of factors contribute to the stagnation of local blood flow. As hyperaemia develops, the capillaries and venules become abnormally permeable to the escape of fluid, resulting in increased blood viscosity, with increased red cell packing producing further resistance to flow and so the outflow of blood from the local site is further impeded (Atherton **et al**, 1973).

b) Alteration of Vascular Permeability

Increased vascular permeability, with the escape of plasma proteins and leucocytes is known as "exudation" and is a major feature of all inflammatory reactions.

The morphologic basis of exudate formation has been studied

extensively in recent years. Under normal circumstances, the endothelium provides the major barrier to permeability. According to Pober, (1990), increased fluid permeability associated with the inflammatory process may be caused by alteration of endothelial integrity in 4 ways. This is suggested to occur by endothelial contraction; cytoskeletal and junctional reorganisation; endothelial cell injury with retraction, lysis and denudation; and endothelial denudation without lysis.

Regardless of which of these contributes to vascular leak, the nett effect is to cause a reduction in shear force and thereby favours leucocyte interaction with the endothelial cell surface.

1.2.3 **Cellular Events**

The massing of the leucocytes, principally neutrophils and macrophages, may well constitute the most important phase of the acute inflammatory response (Wilkinson, 1974). These cells engulf and degrade foreign matter, bacteria, immune complexes and the debris of necrotic cells (Gallin, 1984). The release of their granular contents in combination with the respiratory burst products contribute in a number of ways to the defence response (Weissman **et al**, 1980). But as will be demonstrated later, during these defensive reactions neutrophils also release chemical mediators and toxic radical species that may themselves prolong the inflammatory process and so increase tissue damage.

a) Margination

Since Dutrochet first reported that leucocytes adhered to the vessel and emigrated to the tissues, the interactions between leucocytes and endothelium have attracted great interest (Dutrochet, 1824).

Margination of leucocytes in the blood vessels is thought to depend

on two separate processes: reduced shear forces (Atherton **et al**, 1973) and increased adhesive interaction between the leucocyte and the vascular endothelial cell surface.

The mechanisms by which neutrophils adhere to endothelium during inflammation have been the subject of much attention recently. Such exciting advances allow us to begin to understand these fascinating mechanisms and merit a brief description at this point.

Not only is adherence the first critical step in neutrophil migration, but also in many diseases this may prove to be the site of early inflammatory injury. The adhesion of formed elements of the blood to the endothelium and to each other is mediated by a family of membrane proteins called "integrins" (Weissman, 1989). Three groups have been described: receptors for extra-cellular matrix proteins such as fibronectin and T lymphocyte receptors known as the very late appearing antigens (VLA); platelet surface glycoproteins IIb/IIIa and the vitronectin receptor and the lymphocyte functional antigen (LFA-1) family of leucocyte adhesion molecules.

The LFA-1 family of adhesion molecules includes three heterodimeric glycoproteins which have a common 95kDa beta chain (CD18): LFA-1, Mac-1 (or Mo1, gp165/95 and CR3) and gp(150/90) whose alpha chains have been designated CD11a, 11b and 11c respectively (Sanchez-Madrid **et al**, 1983). The expression of these three molecules varies according to the lineage and the stage of maturation of the various haemopoeitic cells.
In addition to mediating cell-cell adhesion, MAC-1 functions as a receptor for iC3b (CR3) and thereby mediates phagocytosis of opsonised particles (Ross **et al**, 1985). MAC-1 is probably the major neutrophil adhesion molecule involved in heterotypic (neutrophil/endothelium) and homotypic (neutrophil/neutrophil) adhesion. It is constitutively

expressed on the surface of resting neutrophils at a density of 10 000-20 000 molecules per cell (Ross **et al**, 1985)

Several stimuli such as complement components, concanavalin A, formyl-methionly-leucyl-phenylalanine (FMLP), phorbol myristate acetate (PMA) or calcium ionphores increase leucocyte adhesion to endothelium by acting principally on the leucocyte (Tonnesen **et al**, 1984). This is effected by an "upregulation" of receptor expression at the surface of the plasma mebrane by 5 to 10 fold. This increased expression is the result of translocation of preformed receptors from an intracellular source known to co-sediment with the specific granules (O'Shea **et al**, 1985).

Recent studies have shown that in addition to neutrophils, endothelial cells play an active role in all phases of immunologic and non-immunologic inflammation (Wallis **et al**, 1986). This contrasts with the earlier view that vascular endothelium was merely a passive barrier separating the blood circulation from the tissues. With the advent of tissue culture and associated techniques (Jaffe **et al**, 1973) it is now known to be a distributed organ with a wide diversity of function.

In its unique position at the interface between the blood and tissues, vascular endothelium participates in critical haemostatic functions, including the maintenance of a non-thrombogenic surface, regulation of vascular tone and permeability and the modulation of immune function (Cotran **et al**, 1987).

Much of the new information regarding endothelial function in inflammation has come from studies of the effect of cytokines on endothelial cells in culture.

Interleukin 1 (IL-1) and tumour necrosis factor (TNF-alpha) are products of activated macrophages. Lymphotoxin (LT or also known as

TNF-beta) is a polypeptide which is secreted by activated T lymphocytes and has similar tumour killing activities as TNF from activated macrophages (TNF-alpha or cachetin), (Le **et al**, 1987).

IL-1 has two gene products; IL-alpha and IL-beta, which have limited structural homology but have identical biological activities. The TNF gene has also been cloned and has no homology with IL-1 (Pennica **et al**, 1984). TNF and IL-1 have similar inflammatory properties.

These three cytokines have similar effects on cultured endothelial cells. Among these effects, IL-1 was shown to markedly increase tissue factor-like procoagulant activity in both human umbilical vein endothelial cells (HUVEC) and saphenous vein endothelial cells (SAPEC) (Bevilaqua **et al**, 1984). This increase was found to be transient and was maximally expressed 4-8 hours after the cytokine treatment. Proof that RNA and DNA synthesis was required for expression was demonstrated by inhibition in the presence of actinomycin D and cycloheximide respectively. It was later shown that TNF had a similar effect.

In addition, IL-1 and TNF were shown to increase secretion of tissue plasminogen activator inhibitor and reduce the activator itself and so the fine balance between anticoagulant and pro-coagulant activity may be tipped towards fibrin deposition and intravascular coagulation (Nachman **et al**, 1986). Indeed, IL-1 infusion in rabbits was shown to induce fibrin deposition on apparently intact endothelium in-vivo (Naworth **et al**, 1986)

The second notable effect of these cytokines on endothelium is the ability to induce an increase in adhesivity to neutrophils, monocytes, lymphocytes and other cell lines (Pober, 1987). Again this effect can be blocked by RNA and DNA inhibitors and is maximal between 4 and 6 hours, remaining elevated above control levels for up to 24 hours for

neutrophils and monocytes. The development of monoclonal antibodies against cytokine-treated endothelial cells has led to the identification of an endothelial leucocyte adhesion molecule (ELAM-1) which inhibits binding of neutrophils to IL-1, TNF or LT treated endothelial cells during maximal adhesion at 4 to 6 hours (Bevilacqua **et al**, 1987). The ligand on the neutrophil has yet to be identified but it is thought unlikely to be a component of the complex CD11/18 (Luscinskas **et al**, 1988).

In addition to inducing expression of ELAM-1 on endothelial cells; IL-1, LT and TNF also stimulate increased surface expression of ICAM-1 (intercellular adhesion molecule-1, an adhesion molecule present on the surface of fibroblasts, lymphocytes and normal endothelium (Dustin **et al**, 1986). The expression of this molecule on the surface of cytokine-treated endothelium is maximal at 24 hours (in contrast to that for ELAM-1) and is maintained as long as the agonist is present. Recent evidence suggests that ICAM-1 is the ligand for the LFA-1 molecule of the CD11/18 complex (Marlin **et al**, 1987) and that it also may serve as a cell adhesion molecule to bind to lymphocytes, and possibly monocytes and neutrophils to endothelium (Dustin **et al**, 1988).

b) Emigration

This constitutes the mechanism by which motile leucocytes escape from the blood vessels to the perivascular tissues. The importance of the recognition of at least two cytokine-responsive endothelial molecules (ICAM-1 and ELAM-1) in the localisation of neutrophils has just been discussed.

It was only relatively recently, that the requirement for the CD11/CD18 adherence proteins for leucocyte emigration was ascertained.

This was convincingly demonstrated in patients genetically deficient in this glycoprotein complex, termed leucocyte adhesion deficiency (LAD) (Anderson **et al**, 1985).

Further evidence for the requirement of ICAM-1 or CD11/18 complexes for transmigration across endothelium was demonstrated in vitro using using monoclonal antibodies to these complexes (Smith **et al**, 1989; Tonneson **et al**, 1989).

c) Chemotaxis

The movement in the tissues towards the site of injury is termed "chemotaxis". Technically this term describes "unidirectional migration of cells towards an attractant", or more simply, movement oriented along a chemical gradient.

Although there was an early interest in chemotaxis, the development of a micropore filter technique by Boyden, (1962), considerably enlarged the knowledge of substances found to be chemotactic for neutrophils.

Both exogenous and endogenous substances may act as chemoattractants. Some of the notable chemotactic agents for neutrophils include bacterial products such as lipopolysaccharide (LPS) and FMLP; components of the complement system such as C5a, products of the lipoxygenase pathway of arachidonic acid metabolism such as leukotriene B_4 (LTB_4) and hydroxytetranoic acids (HETES), (Vane **et al**, 1987; Cutler **et al**, 1974)

During chemotaxis the bulk of the neutrophils granules are at the front of the cell (Malech **et al**, 1977), and the granule contents are discharged at the leading edge (Cramer **et al**, 1979).

1.2.4 Phagocytosis

Phagocytosis and the release of enzymes by neutrophils and macrophages occur at the site of inflammatory foci.

Phagocytosis involves three processes; first, the recognition and attachment of the particle to be ingested, secondly, engulfment with the formation of a phagocytic vacuole and lastly, the killing and/or degradation of the ingested material.

a) Recognition and Attachment

Most organisms are not recognised until they are coated with naturally occurring "opsonins". The two most common classes of these are immunoglobulin G (IgG; sub-classes 1 and 3), which are presumably naturally occurring antibodies against the ingested particle and C3b, the so-called "opsonic" fragment of C3, which is generated by activation of complement (Henson, 1971). Opsonised particles attach to two corresponding receptors on the surface of neutrophils and macrophages: one for the Fc fragment of the IgG molecules and the other, the C3b receptor (now identified as MAC-1) (Weissman,1989).

b) Engulfment

The plasma membrane of the neutrophil invaginates when engaged by opsonised bacteria or immune complexes. The azurophilic and secondary granules join the newly formed vacuole at its internal border to allow the discharge of their contents (Gallin, 1984).

c) Killing and/or Degradation

Killing involves a multiplicity of mechanisms, all of which are set in motion by degranulation (Zucker-Franklin **et al**, 1964) and the initiation of the respiratory burst (Sbarra **et al**, 1959).

Degranulation describes the process of fusion between the primary phagosome and the granules present in the cytoplasm of the phagocyte.

Table 1(ii)

Granule Constituent of Neutrophils

Class	Primary (Azurophil Granules)	Secondary (Specific Granules)
Microbicidal Enzymes	Myeloperoxidase	
	Lysozyme	Lysozyme
Neutral Serine Proteinases	Elastase	
	Cathepsin G	
Metallo-Proteinases	Collagenase	Collagenase
Acid Hydrolases	N-acetyl-beta-glucosaminidase	
	Cathepsin B	
	Cathepsin D	
	Beta-Glucuronidase	
Others		Lactoferrin
		Cytochrome b
		Vitamin B_{12}-binding proteins

These granules contain substances that participate in the killing and degradation of foreign particles. During this process, these substances, which remain inert as long as the granule membrane remains intact (Lehrer, 1990), are discharged into the vesicle containing the ingested foreign particle. The two granule types may degranulate independantly (Wright **et al**, 1977), and the contents of each are shown in Table 1(ii).

The two main mechanisms by which killing is executed are generally described as oxygen dependent and oxygen independent. The former is an energy dependent process which requires a "burst" of oxygen uptake, glycogenolysis, increased glucose oxidation via the hexose mono-phosphate shunt and production of active oxygen metabolites (Babior, 1978).

d) Oxygen-dependent killing

The NADPH oxidase system is a membrane-associated enzyme complex which participates in the generation of at least three oxygen metabolites; superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$).

The oxidase system is normally inactive but when neutrophils are activated, electrons are moved from the cytosolic NADPH to the oxygen rich fluid at the plasma membrane so that:

$$2O_2 + NADPH \xrightarrow[\text{oxidase}]{\text{NADPH}} 2O_2^{\bullet -} + NADP^+ + H^+$$

two molecules of oxygen accept these electrons. In turn two of the superoxide molecules interact spontaneously in the "dismutation

reaction".

$$2O_2^{\cdot-} + 2H^+ \text{ ------- } H_2O_2 + O_2$$

Although superoxide and hydrogen peroxide may react with other substrates, the dismutation reaction is favoured as the rate of association of superoxide is very rapid (Baboir, 1978).

During degranulation, myeloperoxidase (MPO) is discharged from the azurophilic granules. This alone exerts little bactericidal effect if any, yet in combination with hydrogen peroxide it can oxidise halide ions (usually chloride, as its concentration is 1000 times that of the others) to hypochlorous acid (HOCl) (Weiss, 1989).

$$H_2O_2 + X^- + H^+ \text{ ------} \rightarrow HOX + H_2O$$

(where $X^- = Cl^-, Br^-, I^-, SCN^-$)

The hydrogen peroxide-halide-myeloperoxidase is considered the major anti-microbial system within the neutrophil. Because of the high reactivity of HOCl, it does not accumulate in biologic systems but instead disappears almost instantaneously in multiple reactions with available sustrates (Test **et al**, 1986).

e) Oxygen-independent killing

This mechanism of killing and degradation utilises the lysosomal agents of the granules. Whilst there are over 20 of these, lysozyme, lactoferrin and elastase are of particular importance in this capacity.

Lysozyme is unusual in that it is divided almost equally between the two granule types. Lysozyme is a cationic protein of approximately 14kDa and its enzyme activity is directed against the beta 1-4 glycosidic bond between N-acetylglucosamine and N-acetylmuramic acid residues which

stabilise many bacterial walls (Lehrer, 1990).

Lactoferrin (LF) is a 78kDa, slightly basic glycoprotein which belongs to the transferrin family of iron binding proteins and is contained in the specific granules of the neutrophil. Lactoferrin exerts its bacteriostatic action by tightly binding Fe^{3+}, thus depriving the iron required for synthesis of bacterial components.

Elastase is a highly cationic glycoprotein found in the azurophilic granules. Its activity against bacteria lies in its ability to degrade bacterial cell wall protein and potentiates the lytic activity of lysozyme (Thorne **et al**, 1976).

Although all of the above have some antimicrobial activity, the lysosmal enzymes are generally considered more important for degradation than direct killing.

1.3 TISSUE DAMAGE

From this short description of those processes which constitute the inflammatory response it is clear that the over-riding purpose of the reaction is to permit the survival of the affected tissues and the host as a whole (Stevens **et al**, 1984; Wandell **et al**, 1985).

However, many examples may be cited in which the destruction of the tissue is not due to the damaging stimulus, but to some aspect of the hosts reaction to injury.

Of particular interest in this area, is the role of neutrophils in tissue injury. Its importance as a defence against infection is well known (Wilkinson, 1974; Lehrer, 1990), however the same processes that are so important in the killing and degradation of foreign material, may also act to degrade the surrounding healthy tissue.

The products with potentially harmful consequences in this context are the lysosomal enzymes and oxygen-derived reactive metabolites.

1.3.1 **Lysosomal Enzymes**

There are a number of ways in which the neutrophil, as part of the inflammatory reaction, may effect inappropriate tissue damage.

During phagocytosis granular products may leak into the extracellular space if the phagolysosome system is overwhelmed, thus toxic substances may escape into the space proximal to the cell (Weissman **et al**, 1971). Enzyme release of this kind is termed "regurgitation during feeding" Another mechanism is reverse endocytosis (or frustrated phagocytosis) which occurs when this process is restricted as a result of the cell's adherence to a flat surface, with consequent release of enzymes into the environment. Upon cell death, "cytotoxic release" due to cellular disruption, may result in disgorgement of the granular contents.

Neutrophil granules contain an impressive array of biological weaponry, but three proteolytic enzymes; a serine protease, elastase and two metalloproteinases, collagenase and gelatinase, appear to have the greatest potential as mediators of tissue destruction (Weiss 1989) and is presumably due to their inherent ability to degrade architectural matrix glycoproteins.

Until recently the importance of the two metalloproteinases has been largely ignored, specifically because collagenase and gelatinase are secreted in inactive forms (Weiss **et al**, 1986). Most studies aimed at identifying the activating agent initially involved the use of cell-free systems. As the concept of self-activation grew, contenders for this role included elastase and cathepsin G, but these were found to destroy rather than activate the proteinases (Weiss **et al**, 1986). A more

relevant system for collagenase activation was endorsed by a series of experiments using neutrophils from normal subjects and those from sufferers of chronic granulomatous disease. The results of these pointed to the generation of oxygen metabolites as an absolute requirement for activation. (Weiss **et al**, 1985). Further studies by the same group revealed that normal neutrophils, that were prevented from producing HOCl, were unable to activate collagenase. Not unexpectedly, despite the structural distinctness of gelatinase, the same system was found to trigger the unmasking of the substance's active site. (Peppin **et al**, 1986).

In spite of these findings, most attention has centred on neutrophil elastase and its causal role in tissue destruction. The elastase of neutrophils is a serine protease with a serine residue in position 195 of the enzyme's primary sequence, which contributes a nucleophilic hydroxyl group to attack carbonyl carbons of scissile peptide bonds.

Elastin, the amorphous component of elastic fibre is, as the name suggests, an obvious target for elastase. However its degradative activity is not restricted to this substrate and other important structural targets of the enzyme include collagen types III and IV. Both are cleaved across the helical portions, randomnly in type IV collagen but at a single cleavage site in type III (Mainardi **et al**, 1980), in a manner similar to that of human collagenases. Fibronectin, a major cell-adhesion molecule critical to the organisation of many tissues, is also susceptible to attack (McDonald **et al**, 1980).

In addition to architectural components, many plasma proteins can be hydrolysed by elastase. Among these potential substrates are immunoglobulins (Janoff, 1985), coagulation (Plow, 1975) and complement proteins (Taylor, 1977).

The ability to activate the complement system is of crucial importance in the context of tissue injury, since one of the split products C5a, is a powerful chemotaxin for neutrophils, and therefore may attract further neutrophil migration, thus potentiating the response (Forrest **et al**, 1986).

1.3.2 **Oxygen-derived Metabolites**

Reactive oxygen species, including the superoxide radical ($O_2^{\bar{\cdot}}$), hydrogen peroxide (H_2O_2) and especially the hydroxyl radical ($OH^{\cdot}$), are formed during the process of neutrophil activation (described earlier). These species can react with nucleic acids, proteins and lipids, with resulting damage to the cell membrane or intracellular organelles (Freeman **et al**, 1982). Few studies have successfully demonstrated that the neutrophil uses either $O_2^{\bar{\cdot}}$ and H_2O_2 alone to produce a toxic effect (Weiss **et al**, 1981). There is however evidence that these moieties may play a part in the oxidative inactivation of protective anti-proteases systems and in this way contribute to the extent of tissue damage.

1.3.3 **Regulatory Mechanisms**

Plasma and interstitial fluids contain a series of powerful anti-proteinases including circulating alpha-1-proteinase inhibitor, alpha-2-macroglobulin and secretory leucoproteinase inhibitor, that regulate extracellular neutrophil elastase and prevent the enzyme from attacking extracellular substrates (Fritz **et al**, 1978)

Alpha-1-proteinase inhibitor, (formerly known as alpha-1-antitrypsin), appears to be the most important regulator of neutrophil elastase. This anti-proteinase (MWt 52kD) is synthesised in the liver and is found in the alpha-1-globulin fraction of serum and can

also be detected in broncheolar fluids. Serum levels of the inhibitor rise during inflammation and increased levels of mRNA can be detected in the liver cells during such episodes (Janoff, 1985).

Elastase and its inhibitor form a stable complex when the serine hydroxyl of the active site in elastase attacks the alpha-carbonyl of the methionone residue (at position 358) of the anti-proteinase. Thus under normal conditions, the enzyme is not free to act on other substrates (Carrell **et al**, 1982). The calculated half-life of active elastase in vivo, is only about 0.6msec and Travis estimated that by 3msec all activity should be inhibited (Travis **et al**, 1983).

It would therefore seem unlikely that neutrophil elastase would be present in concentrations sufficient to overwhelm the vigorous protective anti-proteinase shield.

Strong evidence that neutrophils may not only overcome the anti-elastase defence but release their granular contents with ensueing tissue injury, was provided by Opie as early as 1922 (Opie, 1922). He noted that purulent fluids from sites of inflammation contained free neutrophil enzymes which were capable of degrading a variety of native proteins at neutral pH. These observations pointed to the saturation of the natural inhibitory systems, thus permitting the free enzyme to cause tissue damage.

1.3.4 **Circumvention of Regulatory Mechanisms**

A number of suggestions have been made to explain how elastase in particular may escape regulation by its inhibitors.

Firstly, that elastase may be released in quantities that result in saturation of the anti-proteinase shield; secondly, that the enzymes are released in close proximity to the site of damage, thus creating a

microenvironment of high local concentration of enzyme whilst excluding inhibitors (Campbell **et al**, 1982) and lastly, the release of elastase into areas where the inhibitors have been inactivated by oxidation (Weiss,1989). The latter theory is based on evidence that the methionone residue (358) at the reactive site of the anti-proteinase is sensitive to oxidation. Free radical species generated by activated neutrophils have been shown to oxidise this residue with a consequent reduction in the association with elastase by 2000 times, thus extending the half-life of free elastase to 1.2 seconds and its potential to induce tissue destruction.

1.4 NEUTROPHIL INVOLVEMENT IN INFLAMMATORY DISEASE

The neutrophil's ability to release agents that can damage and destroy connective tissue is implicated in the pathogenesis of an increasing number of non-infectious diseases (Weissman **et al**, 1980; Malech **et al**, 1987; Weiss 1989).

For example the association of the destruction of the joint with neutrophil infiltrates has been shown in diseases of the joints such as rheumatoid arthritis (Weissman **et al**, 1984) and gout (Malawista **et al**, 1977).

Neutrophils have also been implicated in the pathogenesis of respiratory diseases and include adult respiratory distress syndrome (ARDS) (Stevens **et al**, 1984) and emphysema (Janoff, 1985).

The pathogenisis of several skin disorders in association with neutrophil infiltration has also been noted eg. in psoriasiform dermatoses (Ragaz **et al**, 1979), autoimmune bullous dermatoses (Glinski **et al**, 1985) and pyoderma gangrenosum (Hickman, 1983).

In addition to the presence of neutrophils at increased density in specific sites associated with the disease process, detection of increased levels of neutrophil degranulation products, particularly neutrophil elastase, indicating a state of neutrophil activation (Plow, 1982), have been reported in non-inflammatory diseases.

In Edinburgh, I have contributed to work detecting increased levels of neutrophil elastase (measured in patient plasma), in patients with diabetes mellitus (Collier **et al**, 1989), ischaemic heart disease and severe hypertension (**Jackson et al**, 1991) and Wegeners granuloma (Wathen **et al**, 1987). Others have also shown increased levels in ischaemic heart disease (Mehta **et al**, 1989) and pregnancy-induced hypertension (Greer **et al**, 1989)

Free radical species, which may be derived from neutrophil activation, have been implicated in the involvement of injury to the skin, intestine, pancreas (McCord, 1985) and also in inflamed rheumatoid joints (Woodruff **et al**, 1986).

1.4.1 **Neutrophil Involvement in Acute Myocardial Infarction**

Recently, studies in animal models of experimental myocardial infarction, have shown that neutrophils and their products of activation may play a part in extending myocyte injury. The following details a few of these studies which illustrate how tissue injury, associated with myocardial infarction, may evolve.

1.4.2 **The Myocardium as a Source of Chemotaxins**

The role of the neutrophil as a contributor to myocardial infarction after chemoattraction was suggested initially in 1971 by Hill (Hill **et al**, 1971).

This possibility was confirmed by Pinckard and co-workers (1973) who, using histological techniques, demonstrated complement activation, one of the most important sources of inflammatory mediators, in animal models of infarction after 3 to 6 hours of ischaemia.

The importance of complement activation was confirmed when cardiac lymph drained from ischaemic myocardium was found to contain molecules of subcellular origin bound to C1q which could activate the complement cascade (Rossen **et al**, 1988). Later studies by this group indicated that cardiac lymph collected after 90 minutes of coronary occlusion in dogs, was not only chemotactic for neutrophils but could also increase the expression of receptors that mediate neutrophil adhesion (Dreyer **et al**, 1989).

In vitro models using tissue culture of myocytes have also provided evidence for the production of substances that are chemotactic for neutrophils under conditions of hypoxia (Friedman **et al**, 1986).

In addition to the myocyte, it was shown that endothelium may also present a potential source of chemotaxins. In vitro treatment of human cultured endothelial cells with IL-1 or TNF-alpha, was found to induce synthesis and secretion of a factor, similar in structure to human monocyte-derived neutrophil activating factor (NAF) (Strieter **et al**, 1988), which is chemotactic for neutrophils.

1.4.3 **Assessment of Factors Influencing Infarction Size**

a) Complement Depletion

The essential requirement for complement, particularly C3 and C5, in neutrophil recruitment and extension of tissue damage in ischaemic myocardium was shown in rats (Hill **et al**, 1971) and also in baboons pretreated with cobra venom factor (Crawford **et al**, 1988). The nett

effect of complement depletion in both studies was a reduction in the amount of myocardium that eventually infarcted.

b) Neutrophil Depletion

Romson demonstrated that a reduction in infarct size could be effected in dogs rendered neutropenic by infusion of rabbit antisera to canine neutrophils (Romson **et al,** 1983). Results indicated that by reducing the neutrophil population by approximately 77%, there was a 43% reduction in the infarct size compared to dogs infused with either saline or non-immune sera.

Several other studies, including that of Mitsos **et al** (1986) and Jolly **et al**, (1986), also measured infarct size in dogs treated with neutrophil antisera. It was shown in the former study, that the control group infarct size was 46 % while the antibody-treated group was reduced to 31% after 90 minutes occlusion and 6 hours reperfusion. Furthermore, lengthening the reperfusion period to 24 hours still resulted in a significant reduction of infarct size (Jolly **et al**, 1986).

c) Neutrophil Inhibition

Similarly, administration of prostaglandin inhibitors have contributed to the understanding of mechanisms of tissue injury.

Ibuprofen, a non-steroidal inflammatory drug, was shown to reduce infarct size in dogs (Romson **et al**, 1982) and prompted Romson to study how ibuprofen affected the influx of ^{111}Indium-labelled platelets and neutrophils into ischaemic heart tissue. Infarct size was reduced significantly by ibuprofen treatment, which was coincident with a 67% reduction in myocardial neutrophil uptake. Platelet infiltration was unaffected in this study and was later confirmed by platelet depletion

studies (Mullane **et al**, 1985).

The apparent dichotomy between the action of two anti-inflammatory agents, ibuprofen and aspirin, was clarified by Flynn **et al**, (1984) who assessed the effects on infarct size in a feline model and on neutrophil function in-vitro. While ibuprofen administration reduced infarct size and inhibited neutrophil function, aspirin was ineffectual on both counts, thus strenghthening the case for neutrophils augmenting myocyte damage.

Several other therapies for reducing infarct size have been investigated. Early studies on the administration of prostacyclin (PGI_2) as a means of limiting myocardial tissue death suggested that its beneficial effects lay in its ability to reduce collateral blood flow to the ischaemic tissue (Jugdutt **et al**, 1981), however this effect was shown to occur in the absence of an increase in blood flow during occlusion (Melin **et al**, 1983). The possible mechanisms by which PGI_2 produced the reduction in infarct size were evaluated by Simpson **et al**, (1987). Intravenous administration of prostacyclin and a PGI_2 analogue (SC39902) were compared in dogs. Both gave a depression in blood pressure but only PGI_2 reduced the extent of myocardial injury. Furthermore when tested in vitro, PGI_2 was found to inhibit neutrophil activation. Therefore the cardioprotective effects of PGI_2 may also be attributed to inhibition of neutrophil function.

d) Neutrophil Adherence Blockade

The action of cytokines, such as IL-1 released from activated macrophages, allow the endothelium to actively participate in the inflammatory process. Although cytokines are thought not to injure endothelium directly, they may help to effect a predisposition to injury

from other stimuli. An elegant study on cytokine-treated rat pulmonary artery endothelium established that pretreatment of endothelium with either IL-1 or TNF rendered it more susceptible to injury by C5a and PMA (Varani **et al**, 1988). It therefore seems likely that endothelial activation and injury may co-exist in vivo.

Although the specific importance of the induction of ICAM-1 and ELAM-1 in the context of myocardial ischaemia is not yet fully understood, studies inhibiting the activity of the MAC-1 (Mo1 or CD11b/CD18) complex on the neutrophil surface (Simpson **et al**, 1988) serve to illustrate the significance of the adhesion reaction of neutrophils in the inflammatory response associated with myocardial infarction. Dogs treated with antibody (904), which binds to the leucocyte adhesion promoting glycoprotein (MAC-1, Mo1, CD11b/CD18), 45 minutes into the 90 minute period of coronary artery occlusion, resulted in an absolute reduction in neutrophil accumulation with a consequent 46% reduction in the mean infarct size. Moreover this effect could not be accounted for by differences in arterial blood pressure, heart rate or neutrophil count.

1.5 FREE RADICAL PRODUCTION IN ACUTE MYOCARDIAL INFARCTION

Because free radicals are by their nature highly reactive species and therefore difficult to detect, it is customary to measure alteration of substrates upon which these moieties may act, and so employ indirect detection.

In special situations electron spin resonance spectroscopy (ESR) can be used to detect free radicals directly in quick-frozen myocardium, or indirectly, employing spin-trap agents which react with the free radicals to form stable adducts.

Using ESR, several groups (Garlick **et al**, 1987 and Zweier **et al**, 1987) have detected a "burst" of free radical activity in isolated perfused hearts in dogs during ischaemia and particularly during reperfusion.

1.5.1 Potential Sources

The production of free radical species in vivo occurs not only in pathologic conditions, but also in the course of normal metabolism and therefore many sources of these species exist.

The three main sources thought most likely to contribute to free radical production during ischaemia are the electron transport system, xanthine oxidase and activated leucocytes.

a) Electron Transport

The majority of oxygen metabolism occurs via tetravalent pathways but 1 to 2% is known to be carried out univalently, thus resulting in generation of small quantities of superoxide and other radicals (Boveris **et al**, 1973). Under ischaemic conditions the percentage of mitochondrial "leak" increases (McCord, 1988).

b) Xanthine Oxidase

The native form of the xanthine enzyme is the dehydrogenase (or Type D), although healthy tissues contain approximately 10% of the enzyme as the oxidase (Type O). During ischaemia, conversion to the oxidase occurs by limited proteolysis (Battelli **et al**, 1972) which, upon reoxygenation, utilises the substrate hypoxanthine to form uric acid, with attendant superoxide anion generation.

C) Leucocytes

As discussed in detail earlier in this chapter, leucocytes constitute a potent source of free radical species through activation of the

membrane-associated NADPH system.

1.5.2 **Free Radicals in Myocardial Infarction**

Although free radical activity is believed to potentiate tissue damage during ischaemia, it is particularly upon reoxygenation that the majority of the injury by this stimulus is considered to occur. Consequently models of reperfusion have provided a valuable insight into the role of these species in myocardial injury.

Reperfusion of reversibly injured myocytes prevents cell death (Braunwald, 1985) and it is also generally accepted that cells salvaged thus, require time to recover structurally and functionally (Jennings **et al**, 1985). In recent years it has been proposed that myocyte death, myocardial "stunning" and arrhymias may not be caused directly by the metabolic events just prior to, but may result more from some deleterious effect of reperfusion. Therefore "reperfusion injury" generally refers to that injury that may not have been expected to occur, except as a result of the reintroduction of the cardiac circulation (Lucchesi **et al**, 1989).

1.5.3 **Interventive Therapies**

The assessment of agents that prevent formation of free radical species eg. allopurinol or oxypurinol; "scavenge" free radicals eg. superoxide dismutase alone or in combination with catalase, or inhibit neutrophil function eg. prostacyclin and ibuprofen in animal models of infarction have helped to ascertain whether a reduction in free radical production also limits infarct size.

While there have been a considerable number and diversity of studies designed to determine free radical involvement in tissue injury

extension, results have often been contradictory as illustrated below.

One of the first studies to show that superoxide dismutase administration in dogs limited infarct size was published by Jolly **et al**, (1984). The results from several subsequent studies; that of Chambers **et al**, (1985), Werns **et al**, (1985) and Nakazawa **et al**, (1988) concurred with this view. In contrast, in later studies superoxide dismutase failed to reduce infarct size (Ambrosio **et al**, 1986; Klein **et al**, 1988; Richard **et al**, 1988).

Similarly, studies on the action of inhibitors of xanthine oxidase, viz allo- and oxy-purinol have provided conflicting views of the effectiveness of these treatments in reducing infarct size. Allopurinol administration was shown by Chambers **et al**, (1985) to limit infarct size, whilst this was disputed in a similar study by Reimer **et al**, (1985). Likewise, opposing data have been reported on the use of oxypurinol (Werns **et al**, 1989; Puett **et al**, 1987). The most important determinants of the variability of results from these studies was suggested by Reimer to be due mainly to "the dose or manner of administration of therapeutic agent, animal species, and experimental protocol, including the duration of occlusion and reperfusion" (Reimer **et al**, 1989).

In spite of this, a substantial body of evidence to support free radical production after ischaemia exist (Burton **et al**, 1984; Zweier **et al**, 1987; Garlick **et al**, 1987). The doubts that persist on the exact role of radicals in myocardial ischaemia mean that although attractive, much work must be completed before interventions may be used confidently in man.

1.6 ASSESSING NEUTROPHIL INVOLVEMENT IN VIVO

Although animal models have explored the factors believed to extend tissue injury, evidence of abnormal neutrophil behaviour in man is limited by observational difficulties. While the use of post-mortem matter is possible in some cases, this material provides a restricted "snap-shot view" of the pathologic process at the time of death.

Neutrophil behaviour can be studied in vivo by following their uptake into sites of inflammation using radiolabelled autologous neutrophils.

Alternatively products of neutrophil activation and end-products of free radical activity can be measured in serum or plasma.

The following section reviews the methods available for assessing neutrophil and free radical involvement in the extension of myocardial injury and justifies their use in this study.

1.6.1 Radiolabelling of White Blood Cells

Animal studies, particularly those performed in canine models, have shown that experimental myocardial infarction may be imaged with radiolabelled neutophils. A preliminary report demonstrated infiltration of ^{111}Indium labelled leucocytes administered 24 hours after experimental myocardial infarction, into infarcted myocardium in dogs at 72 hours after coronary occlusion (Weiss **et al**, 1977). Later these observations were extended by Thakur and co-workers (Thakur **et al**, 1979). This study, again in dogs, indicated that discrete uptake of radioactivity could be imaged, 1-4 days after infarction, but not after 5 days. Furthermore, uptake was maximal in the zones of lowest flow and occurred 24 hours post-infarction in the epicardium and at 72 hours in the endocardium.

Following these encouraging animal studies methods for labelling

neutrophils to follow the acute inflammatory response to myocardial infarction in man have been developed.

Several radionuclides have been used to label leucocytes but as the neutrophil is the principle cell of the response, it is important to distinguish methods that label any white cell from those that label neutrophils.

A radioactive agent which is "ideal" for labelling cells should be specific for the cell type, should neither elute from cells after labelling ex vivo or in vivo, should cause little or no radiation damage, should emit gamma radiation suitable for external detection and lastly should have a half-life suitable for use in clinical studies.

Several radioisotopes have been assessed for cell labelling. The first gamma-emitting compound used to label leucocytes was chromium-51 (^{51}Cr) in the form sodium chromate. It was not ideal since it was not specific for neutrophils, had low labelling efficiency and was also found to accummulate in the reticulo-endothelial system of the liver and the gastrointestinal tract in vivo (Eyre **et al**, 1970).

Gallium-67 (^{67}Ga) has also been used to detect sites of infection and inflammation, either by direct administration to the patient or as a leucocyte labelling agent. The efficiency associated with gallium labelling of leucocytes is low and variable and also the associated radiation burden is high (Lantier **et** al, 1980).

Although ^{99m}Technetium is an ideal imaging agent at the time of this study effective methods of labelling leucocytes with ^{99m}Technetium

McAfee (McAfee **et al**, 1976) showed ^{111}Indium chelates to have physical characteristics superior to tracers previously used for cell labelling as it emits two gamma photons 173keV (84%) and 247keV (94%) which are suitable for external detection by gamma camera imaging. A half-life of 67 hours allows studies to be performed over a period of a few days without having to administer large quantities of radioactivity but not so long as to impart prolonged radiation to the patient after the study is completed.

Indium can be complexed to a number of ligands. Over the years a number have been developed such as acetlyacetone and tetraphenylporphyrin (McAfee **et al**, 1976), but two ligands, oxine (Dewanjee **et al**, 1981) and tropolone (Danpure **et al**, 1982) have proved most useful for use in clinical studies.

Oxine (or 8-hydroxyquinoline) is a lipophilic chelating agent and bacteriostatic, used for many years as a topical antiseptic, fungicide, antiperspirant and spermicide (McAfee **et al**, 1984). It forms 3:1 complexes with trivalent cations like indium (stability constant 10^3) or

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did not exist (McAfee **et al**, 1984). Since the completion of these studies a lipid soluble agent, Hexamethylpropylene amine oxime (HMPAO), formerly used to assess regional cerebral blood flow (Ell **et al**, 1985), has been used to label mixed leucocyte populations. Although this leucocyte labelling method has been used successfully to image inflammatory lesions (Peters **et al**, 1986), there have been no reports of its use in the detection of acute myocardial infarction.

iron, with a nett charge of zero. Due to its lipophilic properties it readily penetrates cellular or bacterial membranes. Once intracellular, the complex is thought to dissociate, with indium binding firmly to the nuclear and cytoplasmic proteins while oxine remains diffusable (Thakur **et al**, 1977).

Since oxine forms complexes with plasma transferrin, the cells must be resuspended in saline to allow efficient labelling. In contrast, tropolone (or 2-hydroxy-2,4,6-cycloheptatiene-1-one), another lipophilic chelating agent, which also forms a 3:1 complex with indium, is suitable for cell labelling in plasma (Saverymuttu **et al**, 1983).

The relative merits of using the tropolone or oxine ligand of indium-111 for leucocyte labelling remains controversial. In vitro tests of cell function, after labelling with tropolone and oxine, have produced variable results (Zakireh **et al**, 1979; Haslett **et al**, 1985). Kinetics studies of neutrophils labelled with ^{111}Indium tropolone showed a rapid passage of the cells through the lungs indicating a "healthy" cell population and was attributed to cell labelling in plasma (Saverymuttu **et al**, 1983).

In spite of this, since ^{111}Indium-oxine was first proposed as an agent for labelling leucocytes (McAfee **et al**, 1976), it is still the most widely used method for cell labelling and imaging inflammatory processes.

The main disadvantage of the use of ^{111}Indium-oxine, as with those also discussed, is that it is a non-specific label and therefore the cell of choice must be isolated prior to labelling. Even for labelling mixed leucocyte suspensions, the bulk of the erythrocyte population must be removed as they are around a thousand times more numerous than the white cells in whole blood. Isolation of neutrophils from other

leucocyte types, particularly the lymphocyte, is also desirable since there are reports that radiolabelling this cell type may have several detrimental effects including induction of severe chromosomal aberrations (ten Berge **et al**, 1983).

1.6.2 **Techniques of Isolating White Blood Cells**

Simple centrifugation of anticoagulated whole blood results in the formation of a "buffy coat", but as only around one third of the total leucocyte population are recovered by aspiration of this layer (Roy **et al**, 1971), alternative methods to improve this have been investigated.

Erythrocyte sedimentation is the most widely used method of obtaining 'mixed' leucocyte suspensions. In 1968 Boyum, developed a simple method of hastening the spontaneous settling of red cells at unit gravity by the addition of an erythrocyte clumping agent. Several such agents including 2% methylcellulose, 6% dextran and 6% hydroxyethyl starch have been used successfully for this purpose (Pfeiffer **et al**, 1982; Danpure **et al**, 1982; Segal **et al**, 1978).

To further purify the leucocyte-rich plasma suspension obtained from erythrocyte sedimentation of whole blood, density gradient (or Isopycnic) centrifugation is often employed. This method of cell separation is governed by Stokes' Law, which states that the rate of sedimentation in a centrifugal field is zero when the cell encounters a medium of equal density. When isolating cells on discontinuous density gradients, the cells migrate until they reach the interface of a solution equal to or greater than their own density; hence, cells of different densities come to rest at different depths.

Since Boyum (1968) showed that Ficoll (produced by the co-polymerisation of sucrose molecules with epichlorohydrin to give a

polysaccharide with an average molecular weight of 40kD) could be used to isolate lymphocytes, researchers have tried many modifications of this for isolation of other leucocyte types.

Perhaps the method most widely used is separation on a density gradient composed of a polysaccharide like Ficoll, in combination with iodinated gradient solutes. Gradients of glycogens, dextrans and other materials have been used, however the commonest is Ficoll (Pharmacia , Uppsala, Sweden), and is generally used in combination with iodinated gradient solutes. Most iodinated compounds used as gradient media have a structure based on tri-iodobenzoic acid to which hydrophilic groups are attached to increase the solubility of these in water eg Hypaque, Isopaque, Metrizamide and Nycodenz (Nyegaard and Co. Oslo, Norway).

A refinement of the Boyum technique was described by English and Anderson (English **et al**, 1974) and involved the separation of leucocyte rich plasma on Ficoll-Hypaque density gradient media comprised of two layers (specific gravity 1.076 and 1.120).

Isolation of cells can be achieved in a single centrifugation step using a single density solution of Ficoll-Hypaque. Ferrante and Thong (1978), found that a mixture of these two components with specific gravity 1.095 caused leucocytes to separate into two bands on centrifugation: lymphocytes in the uppermost and neutrophils in the bottom layer. In later experiments, the density for optimal resolution was determined to be slightly greater, at 1.114g/ml (Ferrante **et al**, 1980).

Percoll (Pharmacia, Uppsala, Sweden) is another media used for density gradient centrifugation. The density of this mixture of silica particles of 15-30 nm diameter, coated with polyvinylpyrrolidone (PVP) can be varied over a wide range by adding balanced salt solutions,

sucrose (0.25M), or even plasma. In one such variation of the use of this material, Percoll is diluted with physiological buffer or platelet poor plasma to three densities: 1.1, 1.0875 and 1.0697 and a tri-layer gradient prepared (Dooley **et al**, 1982). After centrifugation, the neutrophils are found in the middle of the gradient between the lymphocytes and the contaminating erythrocytes.

Until a radio-pharmaceutical with total specificity for the cell of interest is developed, isolation procedures will continue to be required. These methods must therefore result in isolation of a "pure" and "functional" cell population.

In this study two methods for isolating neutrophils from whole blood were compared in terms of efficiency, selectivety, ease and duration of execution and finally on the "activation status" of the cell isolate.

1.6.3 **Clinical Applications**

The results of early attempts to image autologous radiolabelled neutrophils in patients with myocardial infarction were poor. As part of a larger study of inflammatory disease, four patients with acute myocardial infarction were studied using this technique, however uptake of neutrophil-associated ^{111}Indium in the area of infarction was not demonstrated in this small sub-group of patients (McDougall **et al**, 1979).

In a later study, factors which influenced the outcome of imaging the inflammatory response to myocardial infarction were assessed in a larger group of 36 patients. ^{111}Indium activity in the myocardium, representing neutrophil uptake, was evident in 21 of these subjects (Davies **et al**, 1981). Although several parameters, including serum creatine kinase,

infarct site, use of anti-inflammatory drugs, were evaluated, only the age of the patient and the interval between infarction and the time to injection of the radiolabelled neutrophils played a crucial role in the outcome of imaging. These studies addressed the possibility of using labelled neutrophils to demonstrate infarction, however with the development of improved infarct imaging agents such as ^{99m}Technetium pyrophosphate, investigators abandoned the use of labelled neutrophils for the diagnosis of infarction (Klein **et al**, 1978).

My objective was to determine if improved isolation and labelling techniques would allow the study of in vivo behaviour of neutropils in man after myocardial infarction.

1.7 ASSESSING NEUTROPHIL ACTIVATION

There are a number of ways in which neutrophil function and activation status may be assessed. Evaluation of neutrophil locomotion, usually directional (chemotaxis), or interactive reactions such as aggregation or adherence, are often useful tests. Also measurement of products of the neutrophil respiratory burst (superoxide and hydrogen peroxide) and degranulation (lactoferrin, myeloperoxidase, elastase) may also serve as a valuable measure of the activation or competence of the cell population.

Many of these tests are used by specialised haematology laboratories to identify diseases in which neutrophil function is abnormal. For example, defective neutrophil adherence and hydrogen peroxide production is found in leucocyte adhesion deficiency (LAD) and of chronic granulomatous disease (CGD) repectively (Wilkinson, 1974).

The following techniques may be used to assess neutrophil function.

1.7.1 Motility a) Direct Microscopic Examination

The motility of neutrophils may be observed by light microscopy. However as this technique only allows the assessment of a single cell population at a time, dose effect relationships and other multiple preparations of cells are impossible and hence has limited research applications.

b)Agarose Technique

First introduced by Cutler (Cutler, 1974) and later popularised by several groups since then (Nelson **et al**, 1975).

This method allows measurement of directional (to the chemotactic source) and randomn locomotion (vehicle substance) of neutrophils in

agarose towards "wells" containing the test substance. An inherent problem of this method is that the concentration gradient of the test substance will vary during incubation, as the factor will itself diffuse through the agar.

c.) Filter Techniques

Based on the principle of active neutrophil trans-membrane migration towards chemotactic stimuli, the Boyden Chamber (Boyden, 1962) and the raft modification of Addison and Babbage (Addison **et al**, 1976) are the two main methods used. While the raft modification allows multiple determinations, these methods remain lengthy and labour intensive.

1.4.2 Neutrophil Adherence

The adherence reaction of neutrophils may be assessed on artificial substrates such as nylon wool (MacGregor **et al**, 1974) and other plastics (Yakuwa **et al**, 1989; Oez **et al**, 1990). The advent of in vitro tissue culture methods (Jaffe **et al**, 1973), providing a more physiological interface has also allowed the role of endothelial cells in neutrophil adherence reactions to be investigated (Miller **et al**, 1988; Toothill **et al**, 1990).

1.7.3 Neutrophil Aggregation

These tests were derived from existing platelet aggregation technology and are therefore based on the assumption that, like platelets, neutrophils can adhere, aggregate and undergo a release reaction. The two main methods employ light transmission (Yuli, 1984) and electrical impedence (Russell-Smith **et al**, 1982) which relate to the aggregability of the neutrophil population under test.

1.7.4 Products of the Respiratory Burst

The ability of neutrophils to generate these products may be used to indicate neutrophil competence.

a) Superoxide Production

Generation of superoxide may be determined by measuring superoxide dismutase (SOD)-inhibitable cytochrome c reduction (Baboir **et al**, 1973). As many electron donors can reduce cytochrome c, the inclusion of SOD confers specificity to the assay, as only superoxide is destroyed by SOD. Methods of continuous recording changes in absorbance are tedious and permit sequential analysis of a limited number of samples, however the development of a rapid microassay allows simultaneous measurement of hydrogen peroxide (Pick **et al**, 1981).

b) Hydrogen Peroxide Production

Hydrogen peroxide production may be measured by the loss of fluorescence of scopoletin following exposure to hydrogen peroxide in the presence of horseradish peroxidase (HRPO) (Root **et al**, 1975). While this method is very sensitive it requires the availablity of specialised equipment and as a result a simpler method that involves measurement of HRPO-dependent oxidation of phenol red (Pick **et al**, 1980) is generally preferred.

1.7.5 Detection of Neutrophil Granule Release

Detection of extracellular release from neutrophil granules may also serve to indicate the activated state of the cell population. Assays that measure beta-glucuronidase (Mitchell **et al**, 1970), myeloperoxidase (Baggiolini **et al**, 1969) and elastase (Beith **et al**, 1974) provide

evidence of primary granule release. Lactoferrin, degranulation of secondary granules (Quie, 1983), while lysozyme (Shugar, 1952) may be used as a marker of release from both granules.

The two main limitations of the use of most of these methods is that almost without exception these tests require the isolation of neutrophils from whole blood for subsequent testing and, since the process of isolation itself may lead to altered function then the results of such tests must be interpreted with care. Secondly large volumes of blood are usually requisite for neutrophil isolation (40-50ml), and are not ideally suited for repeated assessment over short time intervals, as in the case in myocardial infarction and in other such acute events.

A more convenient approach would allow measurement of markers of neutrophil activation or degranulation in small volumes of blood without requiring neutrophil isolation so that unnecessary disturbance to patient care would be kept at a minimum.

1.7.6 **Neutrophil Elastase Release**

Since elastase is found in high concentrations in neutrophils (4.6ug/10^7 cells; Plow **et al**, 1982) with little additional contribution from other blood elements, and in view of its degradative potential in vivo (Mainardi **et al**, 1980; Taylor **et al**, 1977), this enzyme is considered most appropriate for diagnosing and monitoring inflammatory conditions (Ohlsson **et al**, 1978; Janoff, 1985).

Elastase is synthesised primarily at the promyelocyte stage in the development of the neutrophil and is stored in the cytoplasmic azurophilic granules of the mature cell. The enzyme is a single chain

polypeptide of molecular weight 33kD, with a strongly basic isoelectric point (pH 10 to 11). It has several iso-enzymes and is active at neutral pH (Ohlsson **et al**, 1974).

Elastinolytic proteinases have also been identified in platelets and monocytes as well as in neutrophils (Janoff, 1985).

The enzyme in platelets is present in small amounts (Robert **et al**, 1970) and although not very well characterised, appears to be an elastase distinct from that of the neutrophil on the basis of immunologic criteria (Legrand **et al**, 1975).

Human monocyte elastase is antigenically and biochemically similar to the elastase of neutrophils, but on a per cell basis, monocytes contain considerably less elastase than neutrophils and is located at the plasma membrane (Janoff, 1985).

1.7.7 **Methods for Detecting Neutrophil Elastase**

Methods of measuring neutrophil elastase in its free and complexed form have been developed.

Activity assays measure free elastase by its ability to degrade chromogenic (Hart, 1984) or fluorogenic substrates (Toothill **et al**, 1990) in the absence of inhibitors and therefore are more appropriate for detection in cell-free systems than for use in plasma.

Immunological detection by two different methods, one a standard radioimmunoassay (RIA) (Greer, 1989) and the other, a double antibody enzyme-linked immunoassay (ELISA) technique (Neumann **et al**, 1983) does allow for in vivo detection of neutrophil activation in plasma, sera and other biological fluids.

Plow first described the immunological detection of neutrophil elastase as a marker of neutrophil activation (Plow, 1982). From his

studies he concluded that "normal plasma appeared to contain a basal level of leukocyte elastase-related antigen that could not be attributed to in vitro liberation of the enzyme from leukocytes". Since the presence of calcium ions was found to result in extracellular release of elastase (without cell lysis) the choice of anticoagulant was shown to be an important determinant of the plasma level of elastase. Consequently, citrate or ethylenediamine tetra-acetic acid (EDTA), are recommended as the anticoagulants of choice.

The ELISA technique described by Neumann and co-workers in 1983 was subsequently developed by Merck, Germany. This method measures elastase in complex with its natural inhibitor alpha-1-antiproteinase. The availablity of this prohibitively expensive detection system however post-dated this study.

Therefore despite inherent disadvantages of the use of radioisotopes in detection systems, the radioimmunoassay used here provided a simple and specific assay system which could detect the free enzyme as well as its complexed form. This standard RIA employed polyclonal rabbit antisera raised in-house. The antibody was specific for neutrophil elastase and did not cross-react with either monocyte or platelet elastase. This assay system provided a simple means of assaying large sample numbers with a high degree of sensitivity and precision (Dawes 1987).

1.8 Measurement of Free Radical Species

1.8.1 Free Radicals: Definition

Most chemical reactions occur via heterolytic fission and formation of covalent bonds. However homolytic fission can also take place, thus generating species possessing an unpaired electron, that is a radical.

Electrons in atoms or molecules occupy regions of space known as "orbitals". The maximum number of electrons is two and these spin in opposite directions. Therefore a free radical may be defined as " any species capable of independent existence that contains one or more unpaired electrons" (Halliwell, 1989).

There are a number of ways in which a free radical may be generated from neutral molecules; by photolysis, thermolysis, via redox reactions mediated by inorganic ions, metals and electrolysis that involves one-electron transfers.

Free radicals, thus generated are highly reactive species and have a very short life span; in the order of magnitude of microseconds. These species may react with other molecules in several different ways. The interaction of two free radicals may result in the formation of a covalent bond, shown below;

$$X^{\cdot} + X^{\cdot} \longrightarrow X\text{-}X$$

Alternatively, a free radical species may donate its single electron or may abstract a free radical from another molecule. As a result a non-radical molecule may itself become a free radical species and demonstrates the ability of these moieties to generate free radical chain reactions (Slater, 1984).

The existence and chemistry of free radicals was first studied by

radiation chemists. The products of free radical initiated chain reactions and their applications in every day life are numerous. For example a free radical initiated polymerisation reaction is used to manufacture Teflon^{R}, the non-stick coating for frying pans. Probably one of the most common chain reaction known to modern man is the oxidative breakdown of alkanes in the internal combustion engine.

In the mid 1950's, Gilbert first suggested that damage to living organisms under conditions of high oxygen concentrations, could be attributed to the formation of free radicals (reviewed in Gilbert, 1981). It was not until several decades later that the identification of an enzyme that was specific for the removal of radicals (McCord, **et al**, 1969), now known as superoxide dismutase (SOD), led to general acceptance of the theory that oxygen free radicals may mediate damage to biological systems.

Free radical reactions are vital for the normal operation of a wide spectrum of biologic processes. Endogenous sources of free radicals include those which are generated and act intracellularly, as well as those formed within the cell and released into the surrounding environment. Sites of free radical generation encompass all cellular constituents including the electron transport system in the mitochondria and peroxisomes. Exogenous sources include tobacco smoke, certain pollutants and pesticides (Slater **et al**, 1984).

Several regulators of free radical activity exist. Superoxide dismutase, catalase and glutathione peroxidase are among the intracellular enzyme systems which control the levels of superoxide and hydrogen peroxide. There are also several naturally occurring agents that directly scavenge free radicals which react to produce less harmful radical species. These antioxidants include vitamin E, vitamin C,

beta-carotene and thiols (Machlin **et al**, 1987).

Under normal conditions these and other regulatory mechanisms maintain the balance between radical production and destruction, but in pathological conditions perturbations of these mechanisms may occur leading to free radical-induced tissue damage.

The reactive nature and lack of selectivety in targetting potential substrates, does not help to confine free radical activity in vivo purely to physiological ends. Polyunsaturated fatty acids are found in all cell membranes and are particularly susceptible to oxidation by free radicals (Burrell **et al**, 1989). Damage to proteins (Dilley **et al**, 1984), nucleic acids (Hoffman **et al**, 1984) and lipids (Lunec **et al**, 1979) has been attributed to the action of free radicals.

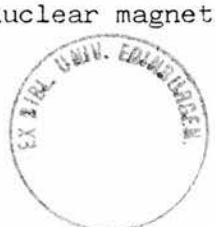
1.8.2 **Methods of Detection**

The short half-life and instability of these species makes their in vivo measurement very difficult and was aptly described by Dormandy as the "diagnostic block" (Dormandy **et al**, 1983).

Consequently, almost all the methods of detection involve indirect measurement of products of free radical interaction.

The exception to this is electron spin resonance spectroscopy (ESR) which allows free radicals to be detected directly in tissues. This technique has been used in the field of pure chemistry for a number of years, but only recently has it been applied to biochemical and biological systems.

The principle of radical detection is based on the application of a magnetic field which reverses the spin of the radicals. Thus ESR measures energy changes that occur as a result of the change of spin direction, not unlike that employed in nuclear magnetic imaging (Symons,



1982). However until this detection system can be adapted successfully for application in humans, as in the case of nuclear magnetic resonance spectroscopy to magnetic resonance imaging, this will be useful only for in vitro studies and animal models (Hess **et al**, 1981; Garlick **et al**, 1987; Zweier **et al**, 1987).

Most other methods for evaluating free radical activity rely on the measurement of alteration to proteins and peptides (Henricksen, 1976), nucleic acids (Henricksen, 1976), and lipids (Iversen, 1985) as a consequence of free radical interaction, or alternatively, the concentration of regulators such as superoxide dismutase (Misra **et al**, 1977), caeruloplasmin (Menden **et al**, 1977) and plasma and red cell thiol levels (Ellman **et al**, 1959).

Methods of measuring alterations of lipid structure under the action of free radical attack, have been the most common means of assessing free radical activity (Dormandy, 1983). Polyunsaturated lipids in cell membranes are particularly sensitive to free radical attack; these molecules consist of double bonds which are interrupted by methylene groups. The hydrogen bonds of these groups are relatively weak and may be easily abstracted by free radical species. After such an abstraction, the resulting sequence of events may proceed in several ways.

In situations of oxidative stress, auto-oxidation may result. This is one of the main free radical reactions in vivo and is the low temperature oxidation of organic compounds by oxygen or oxygen free radical products. Here, oxygen is considered a biradical ($^{\cdot}O\text{-}O^{\cdot}$). The initial stage is commonly the formation of hydroperoxides

eg R-H ---→ ROOH

so is in effect a nett overall displacement, though the actual pathway involves a hydrogen abstraction and O_2 addition.

Alternatively, in vitro experiments have shown that isomerisation of the lipid radical (Iversen **et al**, 1984) through the stabilising delocalised orbitals, may occur in the presence of protein.

All of these products, including the parent molecule, linoleic acid, have the property of diene conjugation and so absorb light at 230-250nm.

These products may be detected by the thiobarbituric acid test (TBA) (Yagi, 1976) or diene conjugation (Iversen **et al**, 1985).

a) The Thiobarbituric Acid Test (TBA)

This is one of the oldest and most frequently used tests for measuring the peroxidation of fatty acids, cell membranes and food products.

The principle of the test is that lipid peroxides react with TBA with the subsequent development of colour. This pink colour is due to the formation of an adduct between TBA and malondialdehyde under acidic conditions which can be measured at 532nm.

The early TBA reaction, while simple and sensitive (Berheim **et al**, 1948), had low specificity. This was improved by including a modification that isolated lipid peroxides from other TBA reacting substances (Yagi **et al**, 1976). Since then further modifications of the method have been made so that measurements on very small samples are possible and also interference by common biological substances such as bilirubin, glucose and sialic acid is reduced (Yagi, 1982).

In attempts to reduce interference in the measurement of the thiobarbituric acid adduct of malondialdehye, several modifications to the detection systems have evolved; using high performance liquid

chromatography (HPLC), (Wong, 1987), fluorometric (Yagi, 1984) and spectrophotometric methods (Bird **et al**, 1984). Nonetheless doubts on its specificity and interpretation of results still persist.

b) Diene Conjugation

Diene conjugated isomerism was studied by Cawood in serum, bile and duodenal juice and identified the major products of free radical activity in a number of systems (Cawood **et al**, 1983). The use of ultraviolet radiation in the presence of protein helped to identify two of the diene products, linoleic acid (18:2(9,12) or (PL-9,12-LA) and the non-peroxide diene conjugate (18:2(9,11) or PL-9,11-LA'). The latter isomer was shown to account for over 90% of the diene conjugation in plasma, tissues and fluids (Cawood **et al**, 1983).

On the basis of this work a method for measuring the diene-conjugated derivative of PL-9,11-LA' in the phospholipid fraction of serum was devised (Iversen **et al**, 1985). This method is based on enzymatic hydrolysis, protein precipitation with final analysis by HPLC and allows measurement of the parent molecule PL-9,12-LA and its isomers.

As with the TBA assay there are critics who question the relevance of the use of the diene conjugation assay since the mechanism by which PL-9,11-LA' originates in man has been questioned (Thompson **et al**, 1985). The dietary composition of fatty acid is known to affect the pattern of fatty acids esterified into serum phospholipids (Holub **et al**, 1978) and may be reflected in tissue and cellular phospholipids, however such an effect has not been reported (Iversen **et al**, 1985).

While each method has its limitations, where several of these methods have been compared there is generally good correlation (Burrell **et al**,

1989). Therefore despite the problems associated with these indirect methods of measurement, both are, and continue to be used widely in the measurement of end-products of free radical interaction in human studies (Yagi 1982; Fink **et al**, 1985; Jennings **et al**, 1986; Plevris **et al**, 1989).

CHAPTER 2

GENERAL METHODS

Procedures used repeatedly throughout this work are described in this chapter. More specific methods are described in the relevant chapters.

2.1 ISOLATION OF HUMAN NEUTROPHILS FROM WHOLE BLOOD

2.1.1 Materials

Mono-Poly Resolving Medium (M-PRM, Flow Laboratories Ltd.)

Phosphate buffered saline, pH 7.4 (PBS)

Preservative-free sodium heparin injection BP, Leo Laboratories Ltd.

2.1.2 Method

All separations were carried out in a laminar flow cabinet using aseptic technique.

From each subject, 60ml of venous blood was collected via a 19G infusion set into a sterile syringe containing 300 units of preservative-free sodium heparin.

In duplicate, 25ml of blood was carefully layered over 12ml of Mono-Poly Resolving Medium (M-PRM), in sterile 50ml polypropylene centrifuge tubes. Any bubbles generated during this transfer were burst with a sterile needle. The tubes were spun at 400g for 45 minutes at room temperature. Differential cell migration during centrifugation results in formation of two distinct white cell bands. The upper is composed of predominantly mononuclear cells and the lower, mainly polymorphonuclear leucocytes, with the erythrocytes forming a pellet at the bottom of the

tube. Figure 2(a).

From the top platelet rich plasma layer, 10ml was collected and spun at 1000g for 10 minutes to provide platelet poor plasma (PPP) and laid aside until required.

The remaining plasma and the mononuclear leucocyte band was carefully removed with a sterile pasteur pipette and discarded. The remaining polymorphonuclear leucocyte band was gently aspirated and transferred to a 40ml volume of phosphate buffered saline (PBS) and spun at 400g. The supernatant was aspirated, the cells resuspended and washed twice in PBS. The supernatant was discarded and the cells resuspended in PBS (10ml) for radiolabelling. An aliquot of the cell suspension was taken for a manual leucocyte count.

2.2 **MANUAL LEUCOCYTE COUNT**

2.2.1 Materials

New Improved Neubauer chamber (0.100mm)

Cover glass (22x25mm) BS748, Weber Scientific International Ltd.

2% acetic acid coloured pale violet with gentian violet crystals (1:20)

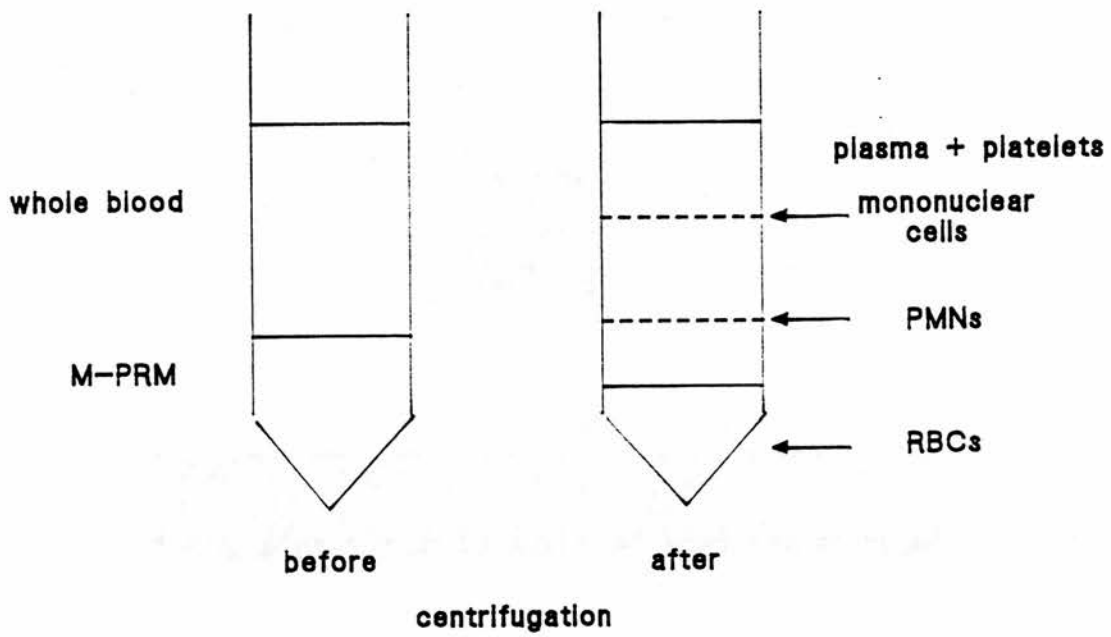
2.2.2 Method

Manual cell counts were performed on whole blood and the cell isolate as follows:

A 1:20 dilution of either whole blood or the leucocyte suspension was made in 2% acetic acid coloured with gentian violet crystals and allowed to mix thoroughly for 10 minutes. Each side of the counting chamber was filled with the cell solution and left undisturbed in a damp atmosphere

Figure 2(a)

Method 1



M–PRM mono–poly resolving medium
PMNs polymorphonuclear leucocytes
RBC red blood cells

for 15 mins to allow complete lysis of contaminating erythrocytes. The leucocytes, with their nuclei stained deep violet/black, were counted over an area representing (0.4 mm^3). This was done on each counting grid of the chamber to improve accuracy.

Cell numbers were expressed as N x 10^9/litre.

2.3 LABELLING NEUTROPHILS WITH ^{111}INDIUM OXINE

2.3.1 Materials

^{111}Indium oxine (1ml, 20-40 MBq), Amersham International plc.

Phosphate buffered saline, pH 7.4, (PBS).

Autologous platelet poor plasma (PPP)

2.3.1 Method

All procedures were carried out using aseptic technique.

The isolated neutrophil population was resuspended in 10ml of phosphate buffered saline (PBS), in a 15ml conical tube. ^{111}Indium oxine solution (1ml) was added dropwise to the suspension and incubated undisturbed for 15 minutes at room temperature. Autologous platelet poor plasma (PPP, 3-4ml), isolated during the separation procedure, was added to the labelled cell suspension and centrifuged at 250g for 10 minutes. The resulting cell pellet was resuspended to 5ml with equal volumes of PPP and PBS. The activities of the cell pellet and supernatant were measured in a radioisotope callibrator (Capintec Inc, ARC 120, Montvale, New Jersey, USA) and from this the labelling efficiency was calculated.

2.4 HUMAN NEUTROPHIL ELASTASE RADIOIMMUNOASSAY

This assay was developed by the Scottish National Blood Transfusion Service (SNBTS)/MRC Blood Components Assay Group, in Edinburgh under the guidance of Dr J Dawes.

This was a standard, specific radioimmunoassay which used rabbit polyclonal antisera raised 'in-house'. The antigen was purified from human neutrophils obtained after leucopheresis. The antibody was specific for neutrophil elastase and did not cross-react with pancreatic, monocyte or platelet elastase. The assay measured neutrophil elastase equally well in its free form or complexed to its natural inhibitors alpha-1-proteinase inhibitor or alpha-2-macroglobulin. Results are expressed as ng/ml.

2.4.1 Sample Collection

Whole blood (5ml), was collected into a tube containing 3.12% trisodium citrate in 5% HEPES buffer, mixed thoroughly and centrifuged at 1000g for 10 minutes at 4°C. The plasma fraction was carefully aspirated and stored at -20°C until assayed, usually within a week of sampling.

2.4.2 Materials

1st antibody	Rabbit anti-neutrophil elastase antibody
2nd antibody	Donkey anti-rabbit immunoglobulin immobilised on Sepharose, made by the Cyanogen Bromide method
Tracer	Human ^{125}I-granulocyte elastase (used at 10ng/ml in Assay Buffer) labelled using the Chloramine-T method
Assay Buffer	0.05M phosphate pH 7.4 (PO_4) 0.6M NaCl 2mM di-sodium EDTA 130ug/ml heparin (porcine), Sigma Chemicals Ltd. 20U/ml aprotonin 2% heat-inactivated horse serum 10% sucrose solution in 0.05M PO_4, 2% heat-inactivated horse serum, 1% Tween 20.
Quality Control	Fresh frozen plasma of assigned value.
Standards	Fresh frozen plasma (20ng/ml), doubling dilutions from neat to 1/128.

2.4.3 Method

All sample dilutions were made in assay buffer (see materials). Plasma samples were assayed at a 1/10 dilution. Doubling dilutions of standard fresh frozen plasma (20ng/ml) from neat to 1/128 were used for the standard curve.

To 50ul of standard or sample, 50ul of anti-elastase antibody (used at 1:6000 dilution) and 50ul of ^{125}I-elastase (10mg/ml) was added and made up to a final volume of 200ul with assay buffer. Samples were thoroughly

mixed and incubated overnight at room temperature. After the addition of donkey anti-rabbit immunoglobulin immobilised on Sepharose (1ml at a 1:6 dilution) and vigorous shaking, the bound complex was separated from free by sedimentation at unit gravity through a 10% sucrose solution. The samples were counted on a NE1600 gamma counter. Results are expressed in ng/ml. Figure 2(b) shows a typical standard curve for this assay.

2.5 MEASUREMENT OF LINOLEIC ACID AND THE NON-PEROXIDE DIENE CONJUGATE

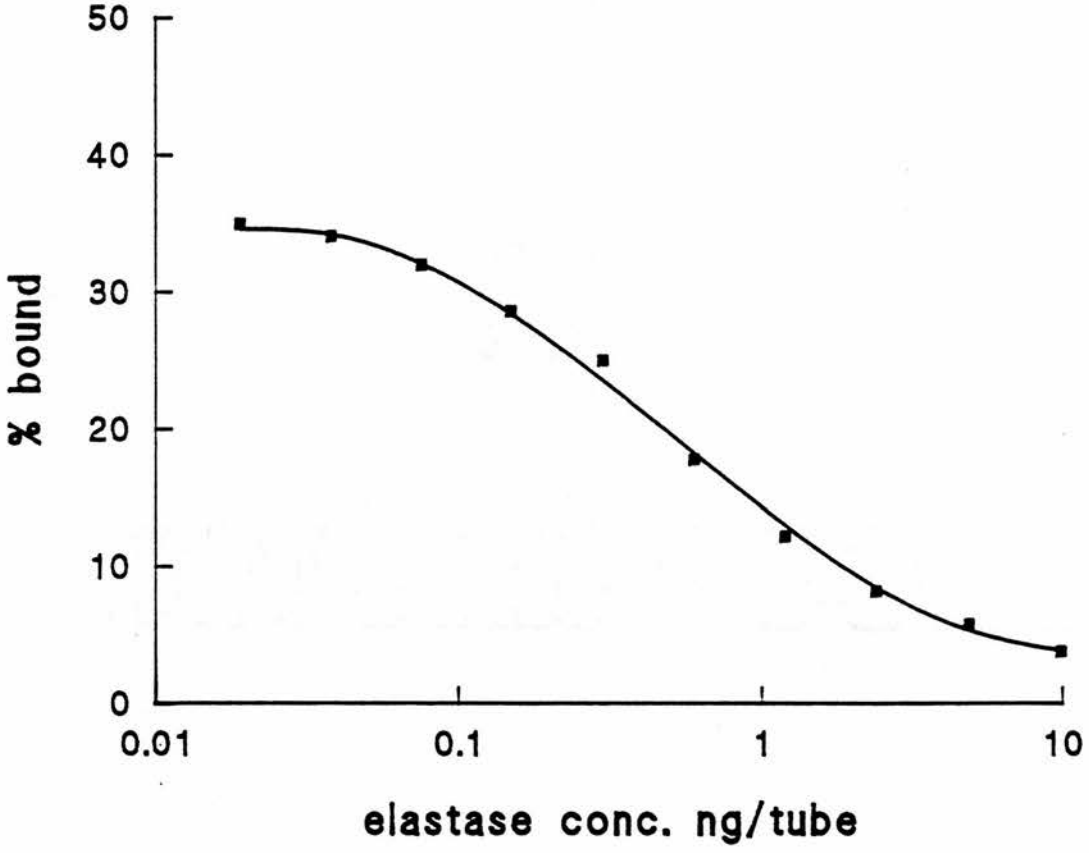
The molar concentrations of linoleic acid (PL-9,12-LA) and and its non-peroxide diene conjugate (PL-9,11-LA') in plasma were measured by high performance liquid chromatography (HPLC), after enzymatic hydrolysis with phospholipase A_2 and solid-phase sample preparation as described by Iversen **et al,** 1985.

Plasma was obtained from heparinised blood samples. PL-9,11-LA' concentration was measured by HPLC, using a sherisorb ODS2 column, a mobile phase of acetonitrile/water/acetic acid (85:15:0.1) at a flow rate of 1.5ml/min, and ultraviolet (UV) detection at 234nm. An internal standard of PL-9,11-LA' trans isomer was used.

Plasma (0.5ml) was incubated for 15 minutes with TRIS buffered phospholipase A_2 to permit enzyme hydrolysis, then the proteins were precipated by the addition of methanol containing the internal standard. After centrifugation, the supernatant was passed through a preconditioned "Bond Elut" column, then, after washing, was eluted with 1ml of propan-2-ol/acetonitrile (2:1). Aliquots of the eluate were

Figure 2(b)

Example of a typical Standard curve for Neutrophil Elastase



injected directly onto the column. The intra-assay coefficient of variation was less than 3.5%. The results were expressed as umol/l.

CHAPTER 3

COMPARISON OF TWO METHODS FOR ISOLATING HUMAN NEUTROPHILS

3.1 INTRODUCTION

Neutrophil labelling with gamma-emitting radionuclides for kinetic studies as well as for imaging inflammatory foci is a useful, yet relatively new technique (Thakur **et al**, 1977).

Because most radioactive agents for cell labelling are non-selective, it is generally necessary to isolate the type of leucocyte of interest.

In this chapter, a comparison is made between two methods for isolating neutrophils from whole blood. The first is a modification of a single step method (Ferrante **et al**, 1980), and uses "Mono-Poly Resolving Medium", (M-PRM) a solution commercially available from Flow Laboratories Ltd.

The second method involves the use of a red cell sedimenting agent, hydroxyethyl starch (Plasmasteril, Fresenius), followed by further separation on a discontinuous density gradient composed of a plasma/Percoll mixture (Pharmacia, Uppsala).

A comparative study of the neutrophil recovery, viability of the isolated cells, degree of contamination by other cellular elements and the time and technical expertise required to execute each of the methods was made.

A total of 15 comparisons were carried out on blood samples from normal laboratory staff. In a sub-group of 10, a differential leucocyte count was made on the cell isolates to assess the purity of the cell population recovered from each method.

3.2 MATERIALS AND METHODS

3.2.1 Blood Collection

Materials

Anticoagulant 0.3ml (300 units) Preservative free sodium heparin (1000 units/ml) Leo Laboratories Ltd.

'19G' Butterfly infusion set

60 ml sterile syringe

Method

All procedures were performed using aseptic technique.

From each normal subject venous blood (60 ml) was collected from the antecubital fossa via a 19G Butterfly infusion set into a sterile syringe containing 300 units preservative-free sodium heparin.

A full blood count was performed on each sample and the remainder split equally between the two techniques to be assessed.

3.2.2 Cell Counts

Materials

New Improved Neubauer Chamber (0.100mm)

Cover glass (22x25mm) BS748, Weber Scientific International Ltd.

2% acetic acid coloured pale violet with gentian violet crystals (1:20)

Method

Manual cell counts were performed on whole blood and the cell isolate as follows:

A 1:20 dilution of either whole blood or the leucocyte suspension was made in 2% acetic acid coloured with gentian violet crystals and allowed to mix thoroughly for 10 minutes. Each side of the counting chamber was filled with the cell solution and left undisturbed in a damp atmosphere for 15 mins to allow complete lysis of contaminant erythrocytes. The leucocytes, with their nuclei stained deep violet/black, were counted over an area representing ($0.4\ mm^3$). This was done on each counting grid of the chamber to improve accuracy.

Cell numbers were expressed as $N \times 10^9$/litre.

3.2.3 Cell Staining Techniques

Materials

Water tight staining baths (2)

Wrights stain, buffered (2.5g/l, pH 6.7 in methanol), Sigma Chemical Co.

Deionised water

Glass slides/coverslips

Method

Clean glass slides were smeared with either whole blood or a few drops of cell isolate and allowed to air dry. These were flooded with 1ml of Wright's stain for 15 seconds and a further 2-3 minutes after the addition of 1ml deionised water. After thorough rinsing the slides were coverslipped in preparation for examination.

Initially the slides were scanned quickly at x10 to provide a general overview. Then using a x50 oil immersion objective differential leucocyte counts were performed by counting at least 300 cells to reduce the inherent error associated with the random distribution of cells.

Differentials were recorded on whole blood and leucocyte suspensions.

3.2.4 Trypan Blue Exclusion Test

Materials

0.4% trypan blue stain (in 0.9% NaCl)

Phosphate buffered saline (PBS) pH 7.4

Haemocytometer and coverslip

Method

This method is based on the principle that viable cells exclude stain from the cell interior and therefore allows enumeration of viable and non-viable cells in a cell sample.

A suspension of the isolated cells ($2\text{-}5 \times 10^5$ cells/ml) was prepared in PBS (pH 7.4). Trypan blue was transferred to a test-tube containing 0.3ml of buffer (PBS) and 0.2ml of the cell suspension and mixed thoroughly. This was allowed to stand for 5-15 minutes. With the coverslip in place, a small amount of the trypan blue-cell suspension was transferred to both chambers of the haemocytometer. All cells, stained or non-stained, in a 1mm centre square and the four corner squares were counted. The percentage of viable cells in the cell preparation was calculated as:

$$\% \text{ CELL VIABILITY} = \frac{\text{Total viable cells (unstained)}}{\text{Total cells (stained and unstained)}} \times 100$$

3.3 Separation of Neutrophils from Whole Blood: Comparison of two methods

3.3.1 Method One : A 'Single Step' Process

This technique involved the use of a single phase medium, Mono-Poly Resolving Medium (M-PRM), produced commercially by Flow Laboratories Ltd. M-PRM is a solution composed of a polysaccharide (Ficoll-400) and a radiopaque contrast medium (Hypaque 85) in a specific ratio to yield a density of 1.114±0.002 at 20°C. This mixture allows the resolution of both mononuclear and polymorphonuclear (PMN) leucocytes into two distinct bands in a single step. See Figure 3(a).

Materials

Mono-Poly Resolving Media (M-PRM), Flow Laboratories Ltd.

Sterile phosphate buffered saline, pH 7.4 (PBS)

Method

Venous blood (25ml) was layered over 12ml of M-PRM in a sterile 50ml Falcon tube and centrifuged at 400g for 45 minutes at room temperature (RT). Differential migration of the cells during centrifugation results in two distinct cell bands and a red cell pellet. The top layer of plasma was recovered by gentle aspiration with a pasteur pipette. The remaining plasma and uppermost cellular band comprising mononuclear leucocytes and platelets was discarded.

The cells in the lower of the two layers were predominantly granulocytes. These were transferred into a 50ml polypropylene centrifugation tube and washed twice in 40ml volumes of PBS. The supernatant was discarded and the remaining cell pellet gently

resuspended in a 10ml volume of phosphate buffered saline (PBS) for evaluation of differential and absolute leucocytes counts and also for estimation of viability.

3.3.2 Method Two : Density Gradient Separation after Red Cell Sedimentation

This method of cell isolation requires an initial step to sediment out the red cell population and is followed by separation of the remaining cells on a discontinuous density gradient. The erythrocyte sedimentation agent used was Plasmasteril and the discontinuous gradient comprised a 42% and 60% mixture of iso-osmotic Percoll and platelet poor plasma (PPP). During centrifugation the mononuclear and polymorphonuclear leucocytes accumulated at the plasma:42% and the 42%:60% interfaces respectively. See figure 3(b).

Materials

Plasmasteril (Fresenius AG, Bad Homburg)

Percoll (Pharmacia, Uppsala, Sweden)

9% NaCl

Phosphate buffered saline, pH 7.4 (PBS)

Method

Plasmasteril (2.5ml) was added to 25ml of whole blood and gently inverted to facilitate thorough mixing. Any bubbles present were burst using a sterile needle. The red cells were allowed to sediment under the action of gravity for 45 minutes at room temperature. The resultant leucocyte platelet rich plasma (LRPRP) was aspirated and transferred to a fresh tube and spun at 200g for 10 minutes to form a leucocyte pellet

in platelet rich plasma. Platelet poor plasma (PPP) was obtained by high speed centrifugation (1000g) of the platelet rich plasma from the previous step.

Iso-osmotic Percoll was prepared by mixing nine parts Percoll (specific gravity 1.13g/ml) with one part 1.5M sodium chloride (9% NaCl). Iso-osmotic Percoll was diluted with PPP to obtain 42% and 60% solutions of Percoll in plasma and 2ml volumes of each were overlayered to construct a two-step discontinuous gradient. The mixed leucocyte pellet was resuspended in PPP (4ml) and carefully layered on top of the gradient and centrifuged at 200g for 5 minutes. The cells from the 42/60% interface were sampled, washed twice in PBS and resuspended in 10ml PBS for differential and absolute leucocyte counts and estimation of cell viability.

3.4 STATISTICS

Results are expressed as mean$\pm$standard deviation. The data for the separation processes using Methods 1 and 2 were analysed using two-tailed Paired Student's t-tests. Values of $p<0.05$ were taken as significant.

3.5 **RESULTS**

3.5.1 Total Cell Recovery

The total neutrophil count and neutrophil count in the cell isolate were assessed manually using a New Improved Neubauer chamber. The cell number recovered from the starting blood volume was expressed as a percentage for each method.

The mean and standard deviation for 15 separations are shown in Table 3(i). The number of neutrophils recovered from 25ml of blood was significantly greater using the M-PRM method ($7.6\pm3.5\times10^{7}$, $p<0.001$) when compared to the Percoll method ($5.3\pm3.6\times10^{7}$). Correspondingly, the percentage neutrophil recovery was also significantly higher using the M-PRM method ($63.3\pm14.0\%$ vs $45.9\pm17.4\%$, $p<0.01$).

3.5.2 Differential Leucocyte Count

The purity of the isolated cell suspension was assessed in 10 of the isolation procedures by performing a differential leucocyte on stained films. A minimum of 300 cells were counted and the numbers of each cell line were recorded and expressed as a percentage of the total number of leucocytes counted. In addition, the number of erythrocytes per 100 leucocytes was also noted. Table 3(ii) shows the mean and standard deviation and the range of the differential counts for 10 simultaneous separations for Method 1 (M-PRM)and Method 2 (Percoll).

The neutrophil percentage present in the cell isolate for Method 1 ($95.2\pm2.5\%$) was significantly higher than for Method 2 ($87.2\pm 4.8\%$, $p<0.001$). In contrast, the lymphocyte presence in the cell suspensions was significantly lower for Method 1 ($2.2\pm1.2\%$) when compared to that of Method 2 ($10.2\pm 4.6\%$; $p<0.001$). The mean percentages of monocytes,

eosinophils and basophils for each method were low and did not differ significantly. The degree of erythrocyte contamination using each method was low at 9.8±5.8 red cells per 100 leucocytes for Method 1 and 8.5±5.3 red cells per 100 leucocytes for Method 2. There was no statistical difference between the two methods.

3.5.3 Cell Viability

The neutrophils recovered by each of the methods showed no significant difference in terms of viability as assessed by the trypan blue exclusion test (Method 1: 98.7± 1.2% vs 98.1±1.6% Method 2)

3.5.4 Time

The separation using the single step was completed approximately 60 minutes less than the discontinuous density gradient method.

Table 3(i)

Mean Cellular Recovery for 15 Separations

		METHOD 1 M-PRM mean±SD (range)	METHOD 2 Percoll mean±SD (range)
Neutrophil	%	95.3±2.5 (91-99)	87.2±4.8 (78.5-91.0)
Lymphocyte	%	2.2±1.2 (0-4.0)	10.2±4.6 (4.0-20.0)
Monocyte	%	1.2±0.6 (0-2.0)	2.1±1.9 (0-6.0)
Eosinophil	%	2.8±1.9 (0-5.0)	2.2±1.1 (0-3.0)
Basophil	%	1.2±0.8 (0-2.0)	1.3±0.7 (0-2.0)
Erythrocyte	%	8.5±5.3 (3.0-20.0)	9.8±5.8 (0-21.5)

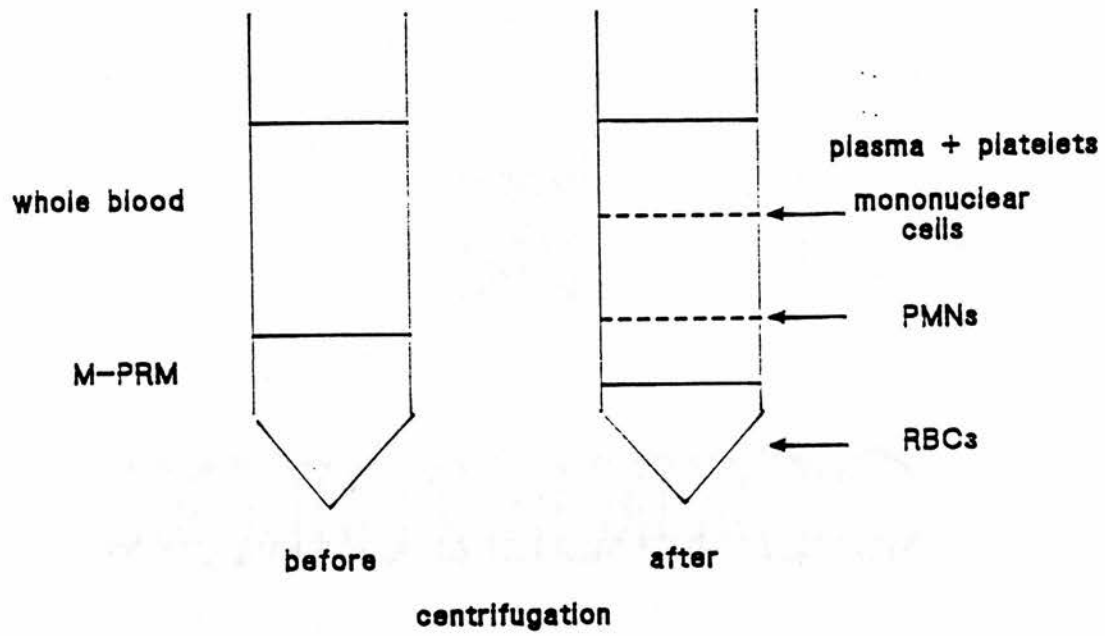
Table 3(ii)

Comparitive Differential Counts for 10 Isolation Procedures

	Method 1 M-PRM	Method 2 Percoll
	mean±SD (range)	mean±SD (range)
Initial leucocyte count (x 10^9/l)	6.6±2.2 (3.2-11.3)	6.6±2.2 (3.2-11.3)
Initial Neutrophil count (x10^9/l)	4.6±1.6 (2.5-8.5)	4.6±1.6 (2.5-8.5)
Neutrophil Recovery x10^7 cells	7.6±3.5 (2.3-16.5)	5.3±3.6 (1.5-16.3)
Percentage Neutrophil Recovery	63.3±14.0 (41-78)	45.9±17.4 (19-76)
Percentage Viability	98.7±1.2 (95.6-99.6)	98.1±1.6 (95.0-99.5)

Figure 3(a)

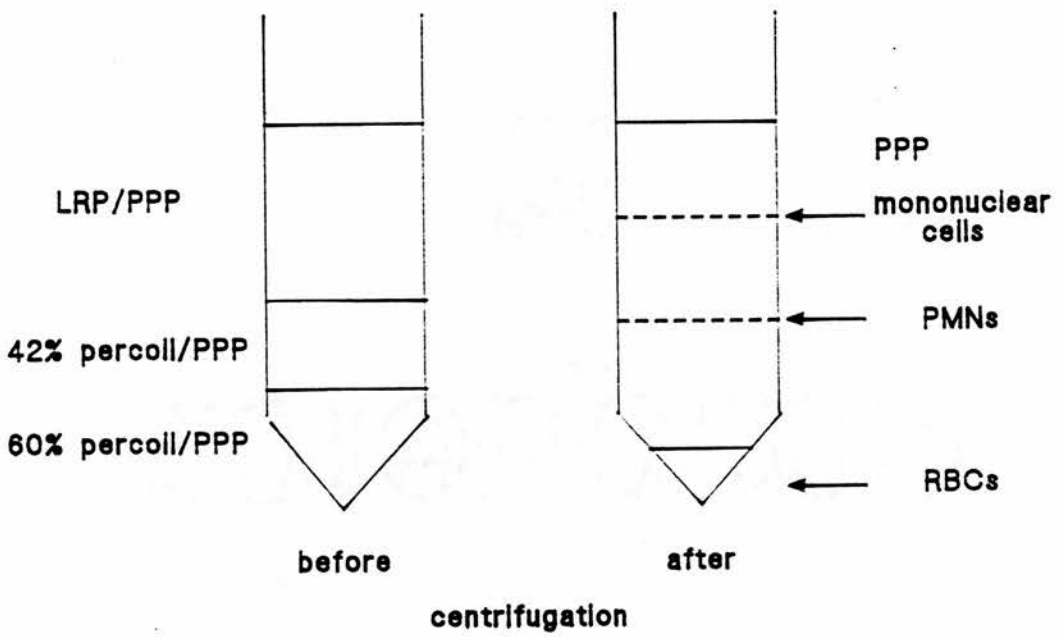
Method 1



M–PRM mono–poly resolving medium
PMNs polymorphonuclear leucocytes
RBC red blood cells

Figure 3(b)

Method 2



PPP platelet poor plasma
LRP leucocyte rich plasma
PMNs polymorphonuclear leucocytes
RBC red blood cells

3.6 **DISCUSSION**

Isolation of neutrophils from whole blood is mandatory for radiolabelling since current techniques allow neither labelling of specific cell types in vivo nor in whole blood in vitro.
Many researchers have long sought a simple means of isolating neutrophils from whole blood while maintaining their viability. All separation techniques are bound to cause some change in the nature of the cell, but until reliable specific in vivo labelling of neutrophils is accomplished, these methods will continue to be used.

A method which would be considered 'acceptable' must therefore fulfil certain criteria. First the method must yield an essentially 'pure' preparation of neutrophils. Most radioisotopes, including ^{111}Indium-oxine will label all cell types in a blood sample. Ideally, the red cells and other cell lines should constitute only a very low percentage of the cell population to minimise competition for the radionuclide. In particular, lymphocyte numbers should be as low as possible, since several studies have shown that labelling this cell type with ^{111}Indium oxine can result in spontaneous release of the label, 25% at 24 hours, and a dose dependent reduction in the cells proliferative capacity in response to several stimuli (ten Berge **et al**, 1983; Balaban **et al**, 1987). Probably the most significant finding is that human lymphocytes after exposure to ^{111}Indium oxine, but not its decayed form, exhibit several chromosomal aberrations including gaps, breaks and exchanges. It is well known that ionising radiation has mutagenic and carcinogenic properties (Ischimari **et al**, 1971). Therefore, where possible, it is advisable to avoid administering ^{111}In-labelled lymphocytes as contaminants of granulocyte suspensions. By preparing an essentially pure isolate of neutrophils these two main problems should

be circumvented.

Secondly, the requirement that the isolated cells are still biologically competent is of paramount importance. Therefore to minimise alteration in the functional and morphological integrity of the isolated cell the separation method ideally should be a short, simple procedure that requires a minimum of "cell manipulation".

Here, two methods were compared in these terms. The single step procedure was shown to result in superior neutrophil isolation, highlighted by a higher total recovery of neutrophils. The cell isolate from this technique produced a "purer" preparation, with a significantly greater proportion of neutrophils and a lower content of lymphocytes. Red cell contamination was low for each method with approximately 8 erythrocytes per 100 leucocytes, which should not present significant problems when using radionuclides such as indium because of the high relative affinity of both of these chelating agents for granulocytes compared with red cells (Weiblen **et al**, 1979).

The trypan blue exclusion test, a crude measure of neutrophil viability showed no difference between methods. The time to complete each method was taken into account and Method 1 was consistently completed 60 minutes in advance of Method 2. The difference was mainly attributable to the red cell sedimentation step required in the latter case and also to the time taken to construct the discontinuous density gradient.

In conclusion, Method 1, the single step procedure using Mono-Poly Resolving Medium, satisfied the main requirements for a cell isolation technique. Its use resulted in good neutrophil recovery, with little red cell and virtually no lymphocyte contamination. The method was rapid and involved less 'handling' of cells, thus reducing the risk of mechanical

damage and requiring less technical expertise.

CHAPTER 4

THE EFFECTS OF SEDIMENTATION AGENTS AND DENSITY GRADIENT MEDIA ON THE NEUTROPHIL

4.1 INTRODUCTION

Chapter 3 detailed a comparison of two methods for isolating neutrophils from whole blood. It concluded that the single step procedure using Mono-Poly Resolving Medium (M-PRM), proved technically easier, could be completed rapidly and resulted in isolation of a 'purer' neutrophil preparation than the method requiring initial red cell sedimentation followed by separation through a discontinous density gradient. However, while the M-PRM method has been proven to be superior in this work, it is relatively new and techniques requiring red cell sedimentation are still used routinely in many laboratories for neutrophil isolation, with slight variations in the sedimentation agents used and composition of the density steps in the discontinuous gradients.

It is thought that the methods and materials used to isolate neutrophils may alter the function of the cells (Saverymuttu **et al**, 1983). As a result there has been considerable interest in the methods for isolating neutrophils (Haslett **et al**, 1985; Saverymuttu **et al**, 1983), and how these procedures may alter the function of the resultant cells.

Ideally procedures for cell isolation should produce little or no cell activation. In 1985, Haslett suggested that trace amounts of bacterial lipopolysaccharide (LPS), found in preparations of Ficoll-Hypaque but not Percoll, reduced the isolated neutrophil's chemotactic responsiveness and increased lysosomal enzyme release upon

stimulation with formyl-methionyl-leucyl-phenylalanine (FMLP).

Lane and co-workers demonstrated that isolated rabbit neutrophils, when radiolabelled and subsequently reinjected, showed reduced recirculation in vivo as a result of in vitro manipulation. In contrast, these cells were shown to exhibit normal behaviour in in vitro tests for chemotaxis or enzyme secretion (Lane **et al**, 1982) and therefore questions the validity of in vitro methods of assessing cell function.

Neutrophils that are 'activated' cells become more 'adhesive' in nature and this may explain delayed transit through the lung capillary bed in vivo (Saverymuttu **et al**, 1983). Cells activated during isolation from whole blood therefore, should not be reintroduced into the circulation either as a qualitative test of cell function or as a diagnostic tool for location of sites of inflammation.

As discussed in Chapter 1 there are many ways to assess cell function. However, the majority of these techniques require cell isolation, the process under scrutiny, therefore making such an assessment difficult.

Immunological detection of the release of neutrophil elastase from neutrophils has been identified as a method of assessing neutrophil activation (Plow **et al**, 1982), and can be measured in blood as well as physiological media. Release of neutrophil elastase is by active secretion, during phagocytosis or upon cell death, and is therefore a sensitive index of its activated state.

In view of the concern and conflicting ideas surrounding the effects of density gradient media and erythrocyte sedimentating agents on neutrophil function, isolation procedures and their effect on neutrophil activation was assessed.

The concentration of neutrophil elastase produced during incubation of whole blood with three erythrocyte sedimentation agents;

methylcellulose, hydroxyethyl starch and dextran and two density gradient media; Mono-Poly Resolving Medium (M-PRM; a Ficoll-Hypaque mixture) and Percoll was measured. Experiments were designed to simulate conditions under which the agents would normally be used.

Neutrophil elastase, as a marker of neutrophil activation, was measured using the specific radioimmunoassay as described in Chapter 2.

4.2 EFFECT OF SEDIMENTATION AGENTS

4.2.1 MATERIALS AND METHODS

Materials

6% dextran 70 in 0.9% NaCl (Pharmacia)

6% hydroxyethyl starch in 0.9% NaCl (Plasmasteril, Pharmacia)

2% methylcellulose in 0.9% NaCl (Methocell E50 Premium, Dow)

Method

From 10 normal subjects, a 40ml sample of whole blood was withdrawn into a sterile syringe containing 200 units preservative-free sodium heparin. A 10ml sample was retained as a control sample and the remaining 30ml was divided equally between three tubes containing a sterile solution of either 6% dextran 70 in 0.9% NaCl (4.0ml), 6% hydroxyethyl starch in 0.9% NaCl (1.0ml) or 2% methylcellulose in 0.9% NaCl (0.4ml). The volume of each of the agents was chosen to give the concentration used routinely for erythrocyte sedimentation. The samples were gently inverted to ensure thorough mixing and then divided in two. One half of the sample was kept at room temperature (21°C) while the remainder was incubated at 37°C. Red cell sedimentation was allowed to proceed for 45 minutes at unit gravity. At the end of this period the supernatants were carefully aspirated with a pasteur pipette and spun at 1500g for 10 minutes at 4°C to render the sample cell-free. The concentrations of neutrophil elastase in the resulting supernatants were assayed using a standard radioimmunoassay (described in Chapter 2). The results were corrected for any dilution caused by the sedimentation agents.

4.3 EFFECT OF DENSITY GRADIENT MEDIA

4.3.1 MATERIALS AND METHODS

Materials

Mono-Poly Resolving Medium (M-PRM),(Flow Laboratories Ltd).

Percoll (Pharmacia, Uppsala).

Phosphate buffered saline, pH 7.4 (PBS).

Method

Whole blood (10ml) was taken from eight healthy volunteers and anticoagulated with sodium heparin (preservative free). This was split into two 5ml aliquots and added to either a 2.4ml volume of Mono-Poly Resolving Medium (M-PRM) or a solution of iso-osmotic Percoll made 51% in phosphate buffered saline (PBS). The ratio of blood to medium was chosen to represent those used for these separation techniques. The samples were placed on a rotary mixer at room temperature (21°C) for 15 minutes to ensure continuous contact between the separation media and whole blood.

The samples were then centrifuged at 1500g for 10 minutes at 4°C. The supernatants were aspirated and re-centrifuged to render them cell free. Neutrophil elastase was measured in an aliquot of the resulting supernatant, as described in Chapter 2. In addition, total lactate dehydrogenase (LDH), as an indicator of cell viability, was measured in the clinical chemistry department by the method of Bryden **et al**, (1973), adapted for use with a centrifugal analyser (Cobas Fara, Roche, Welwyn Garden City, UK)

4.4 **STATISTICAL ANALYSIS**

Results are expressed as mean $\pm$ SEM. The data for the neutrophil elastase levels in the supernatants of the samples after treatment with either sedimentation agents or density gradient media are expressed as ng/ml. Statistical analyses were performed using the Wilcoxon's test for signed ranks. Values of $p<0.05$ were taken as significant.

4.5 **RESULTS**

4.5.1 Effect of Sedimenting Agents:

Table 4(i) and Figure 4(a) show the mean results for neutrophil elastase in ng/ml for the controls and for those samples treated with each sedimentation agent at room temperature (21°C) and at 37°C.

4.5.2 At Room Temperature

The neutrophil elastase levels in the samples treated with 6% hydroxyethyl starch (51.8±7.8 ng/ml; $p<0.01$) and 2% methylcellulose (46.9±6.8 ng/ml; $p=0.01$) were significantly greater than the levels measured in the control samples (32.4±5.0 ng/ml). In contrast, there was no significant difference between neutrophil elastase levels for the controls and samples incubated with 6% Dextran 70, (32.4±5.0 ng/ml). When the effects of the sedimentation agents on neutrophil elastase release within the group were examined, no statistical difference between the effects of treatment of whole blood with either hydroxyethyl starch and methylcellulose was evident. Both agents, however caused significantly more neutrophil activation than Dextran 70 ($p<0.01$ and $p=0.01$ respectively).

4.5.3 At 37°C

Neutrophil activation induced by the sedimenting agents at 37°C exhibited a pattern similar to those seen at room temperature, but of greater magnitude.

Incubation of the samples treated with sedimenting agents at 37°C induced significant neutrophil activation in the supernatants when compared to controls. This was most pronounced in those samples treated with methlycellulose (206.6±43.2 ng/ml; $p<0.01$), with a slightly lower

values for hydroxyethyl starch (186.3±28.6 ng/ml; $p<0.01$) and the least amount of neutrophil activation induced by Dextran 70 (104.7 ± 15.7 ng/ml; $p<0.01$). There was no significant difference between hydroxyethyl starch and methylcellulose.

4.5.4 Effect of Density Gradient Media

The mean results (±SEM) for neutrophil elastase in the sample supernatants after treatment with Mono-Poly Resolving Medium (M-PRM; 19.8±2.0 ng/ml) was significantly lower than for Percoll (25.1±2.7 ng/ml; $p<0.01$), however these results lay within the normal laboratory range for plasma levels (20.8±11.0 ng/ml).

No statistical difference was found between total LDH levels for samples treated with M-PRM (319.5±24.0 U/l) or Percoll (318±50.2 U/l).

TABLE 4(i)

The mean±SEM for 10 samples incubated for 45 minutes at room temperature (21°C) or at 37°C.

	Neutrophil Elastase (ng/ml)	
	RT(21°C)	37°C
6% Dextran 70 (in 0.9% NaCl)	32.4±5.0	104.7±15.7
6% Hydroxyethyl Starch (in 0.9% NaCl)	51.8±7.8	186.3±28.6
2% Methylcellulose (in 0.9% NaCl)	46.9±6.8	206.6±43.2

TABLE 4(ii)

The mean results±SEM for 8 samples incubated with either M-PRM or Percoll

	M-PRM	Percoll
Total LDH (U/l)	319.5±8.5	318±17.7
Neutrophil Elastase (ng/ml)	19.8±2.0	25.1±2.7

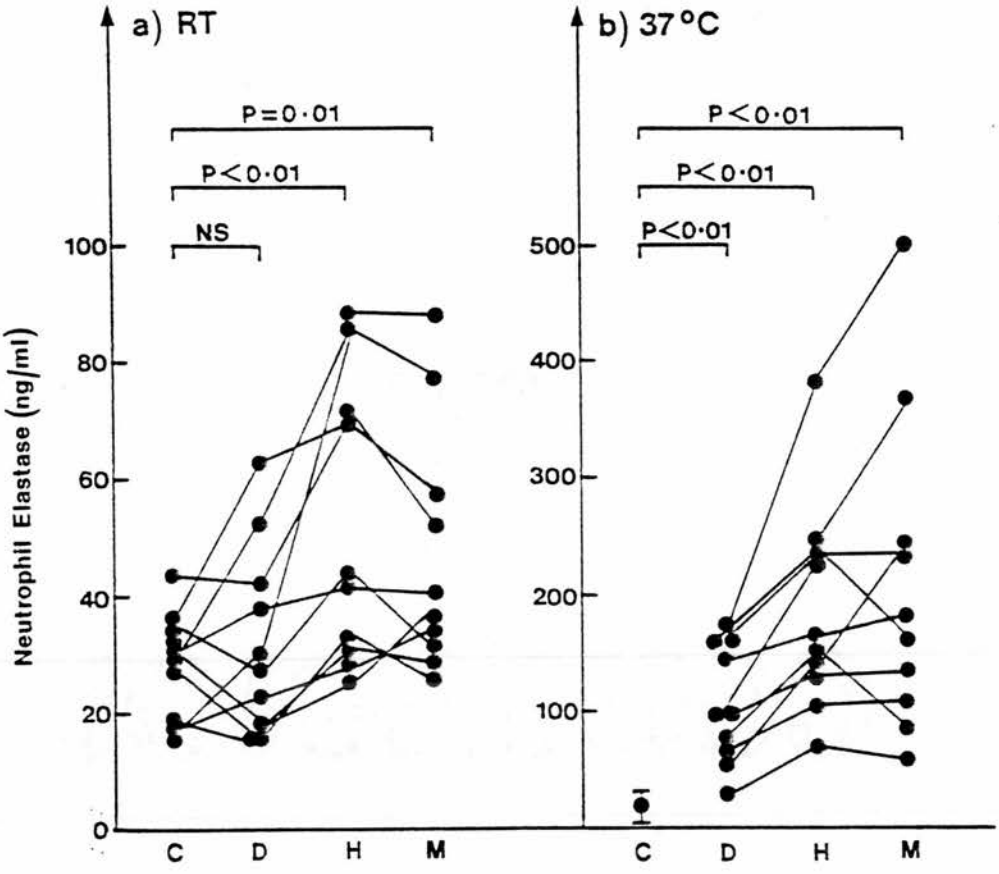


Figure 4(a)

Release of neutrophil elastase in 10 subjects. (a) RT = room temperature of 21°C; (b) 37°C. (C = controls; D = dextran; H = hydroxyethyl starch; M = methylcellulose)

4.6 **DISCUSSION**

That cells may be altered from their 'resting state' by isolation procedures, was demonstrated at the receptor level, in two studies in which the expression of complement receptors on the neutrophil plasma membrane was examined. Fearon **et al**, (1983) using indirect immunofluorescence staining and flow cytometry demonstrated that neutrophils expressed low levels of C3b (CR3) on their cell membrane and that this was unaffected by increasing temperature between 20°C and 37°C. However an 8-fold increase in expression was invoked by increasing the temperature of the isolated cell population. This increased expression, considered in this study to represent maximal expression, was only elicited in cells isolated from whole blood and was thought to relate to the isolation procedure itself. This was confirmed by Berger **et al**, (1984) who, using monoclonal antibodies and flow cytometry, detected both C3b (CR3) and C3bi (CR1) on the neutrophils in whole blood and cell isolates. Cells isolated from whole blood were shown to "spontaneously" increase expression of these complement receptors at raised temperatures, but could be further augmented by chemotactic stimuli suggesting incomplete expression. Since the "spontaneous" expression of the receptors occurred within minutes and in the presence of protein synthesis inhibitors, it was suggested that the receptors were translocated from an intracellular pool, the most likely site being the primary granules. Additionally the specific effect of both Percoll and Ficoll-Hypaque type density gradients on the receptor expression was examined and no difference was found to exist between these.

In contrast, in a study of cell function after cell isolation procedures, these two widely used density gradient media were claimed to have differing effects on the functional state neutrophils. This was

attributed to the presence of the bacterial lipopolysaccharide (LPS) in Ficoll-Hypaque, but not Percoll, (density gradient media on which the cells were isolated) which according to Haslett **et al**,(1985) "primes" neutrophils, rendering them more susceptible to "activation".

As a result a great deal of controversy exists on the relative merits and disadvantages of the use of density gradient media that are available for cell isolation.

Percoll, a colloidal silica coated with polyvinylpyrrolidone and Ficoll-Hypaque mixtures (of which M-PRM is an example) are among the most commonly used agents.

These density gradient media and three red cell sedimentation agents were assessed for their effect on neutrophil activation. The results show that neither Percoll nor M-PRM caused significant neutrophil activation during co-incubation with whole blood. In each case, neutrophil elastase levels measured were within the normal laboratory range (see Chapter 7), although treatment of whole blood with Percoll resulted in elastase levels greater than for M-PRM.

In contrast, two of the three sedimentation agents; methylcellulose and hydroxyethyl starch, when tested at room temperature caused significant neutrophil activation, as did all three at 37°C. Therefore if erythrocyte sedimentation is unavoidable, dextran is the agent of choice and should be carried out at room temperature.

In conclusion, of the methods available for cell isolation, a single step procedure, which does not require red cell sedimentation, is most satisfactory since this minimises possible sources of cell activation.

CHAPTER 5

IN-VIVO BEHAVIOUR OF ^{111}INDIUM LABELLED NEUTROPHILS SEPARATED ON MONO-POLY RESOLVING MEDIUM (M-PRM)

5.1 INTRODUCTION

The processes of isolation from whole blood and subsequent radiolabelling of neutrophils may alter their function. Ideally, cell viability should be checked on all labelled cell preparations before administration to the patient.

As yet, the best assessment of in vivo granulocyte viability appears to be the pattern of cell transit through the circulatory system immediately after administration (Saverymuttu **et al**, 1983).

Activation of the neutrophil population during in vitro processing will be manifested in vivo by a departure from what is considered to be the normal distribution of the cells in the circulation. Much of the early work on granulocytes kinetics was performed with ^{32}DFP or ^{51}Cr labelling (Dresch **et al**, 1975, McMillan **et al**, 1968) in human studies and provided valuable information on the total body granulocyte pool, the rate of production, intravascular life-span and also distribution between the marginal and circulating pools. Normal granulocytes should, after injection, pass rapidly through the lungs and equilibrate between the marginating pools of the liver (usually within 5-15 minutes) and the spleen (in about 40 minutes) (Peters **et al**, 1988).

Several studies have shown that poorly functioning or damaged cells demonstrate lung retention and liver sequestration (Thakur **et al**, 1977; McAfee **et al**, 1980; Saverymuttu **et al**, 1983). This is thought to be a consequence of neutrophil activation since these cells show increased endothelial adherence, and as the lungs present the first microvascular

bed after reinjection, a significant proportion are "held up" here. These cells on release from the pulmonary endothelial surface undergo early non-specific removal into the reticuloendothelial system and is manifested by high activity in the liver. An increase in pulmonary margination under pathophysiological influence eg. that seen in adult respiratory distress syndrome (ARDS), can be differentiated from that due to in vitro damage to the cell population, by the persistence of activity in the lungs compared to that for the circulating pool.

Optimal cell viability therefore is associated with rapid lung transit, rapid spleen uptake, low liver uptake and rapid accumulation within inflammatory loci.

It is therefore essential, as a qualitative measure, that cells prepared by any 'new' isolation or labelling techniques be assessed for lung retention and liver uptake. One method of achieving this is to compare the passage through the lungs of simultaneously injected ^{99m}Tc-labelled red blood cells and ^{111}Indium labelled neutrophils with simultaneous acquisition in the two corresponding energy windows (Muir **et al**, 1984). Using this method the ^{99m}Tc time-activity curve represents the passage of red cells through the lungs. Any retention of ^{111}In labelled neutrophils within the lung vasculature will be demonstrated by a difference in the lung time-activity curves for the two radionuclides. Although dual isotope studies are an elegant solution, they are technically demanding and the gamma camera system available at the time of these studies did not have dual acquisition capabilities.

A simpler single isotope technique was used to image the passage of the labelled neutrophils through the heart and lungs. Time-activity curves for the heart and lung were made following a bolus injection of ^{111}labelled autologous neutrophils. Differences between the curves

represented lung retention. Uptake in the liver and spleen was also recorded.

5.2 SUBJECTS

The kinetics of ^{111}Indium labelled neutrophils were studied in a group of six subjects using this imaging technique. The six individuals all gave informed consent and had no evidence of active lung infection or disease.

5.3 **METHODS**

5.3.1 Neutrophil Isolation and Radiolabelling

The separation and labelling procedures described in Chapter 1 were used to prepare the cells for reinjection.

5.3.2 Imaging

The following imaging technique was used to assess the in vivo kinetics of the ^{111}Indium labelled neutrophils.

Imaging was performed using a large field of view gamma camera (GEC-400T Maxicamera) interfaced to a PDP11-34 computer (Digital Equipment Corporation).

The subject to be studied was positioned below the camera, in the anterior position, to visualise lungs, liver, spleen and heart in the same field of view. It was ensured that the subject remained still throughout the whole procedure.

The autologous ^{111}Indium neutrophil suspension was administered intravenously via a 16G cannula inserted in the right arm. The bolus was flushed through with a 100ml of 5% dextrose. A sequence of images were accumulated over the first 25 minutes of scanning time from the start of

administering the labelled cell suspension. The time frames were of varying length starting with 5 second frames and increased stepwise to 60 second acquisition frames for the latter images. The counts were normalised for frame length.

5.3.3 Analysis of Images

Using each still frame acquired by the Gamma camera/PDP11-34 facility a set of "movie" images were compiled. This allowed the transit of the cells through the heart, lungs and subsequent accumulation in the spleen and liver to be viewed on screen. This sequence was "stopped" at a frame that conveniently allowed clear definition of the organs of interest and using a light pen these were outlined and saved on disc. The counts in each of these areas or regions of interest (ROI), then represented the activity of the neutrophils as they passed through the organs at any given time. The whole field of view showing the maximum count rate was assumed to represent the total activity injected. The count rates from the other regions of interest were expressed as a percentage of the maximum whole field count rate. In this way time-activity curves were created for each organ. Using this method of data analysis, direct comparison of the relative activity in each organ for each subject was possible.

5.4 **RESULTS**

5.4.1 Cellular Recovery and Labelling

Neutrophils were isolated from whole blood in six subjects. The mean neutrophil recovery was 60±15% which resulted in isolation of $19.4 \pm 9.1 \times 10^7$ cells. The neutrophils were labelled with ^{111}Indium-oxine with a mean labelling efficiency 73.3±8.7%. The mean cell-associated dose administered was 20.8±8.2 MBq. Results are shown in Table 5(i).

5.4.2 Imaging

For each subject the absolute counts for each organ are shown in Tables 5(ii) and 5(iii). Table 5(iv) shows the mean indium-111 neutrophil kinetic data obtained from studies in six subjects. The data were expressed as percentages of the maximum count rate achieved in the whole field of view and the time-activity curves for each organ are shown in Figure 5(a).

The mean heart and lung count rates were maximal at 12 seconds at 6.7% and 35.2% of the total activity injected respectively. The count rate over the heart fell to below 3% within the first 90 seconds of imaging and after 5 minutes remained constant at approximately 1.9% for the remainder of the study. The count rate corresponding to the area of lung chosen, fell in a similar fashion to 17% within the first 90 seconds and thereafter at a slower rate, to 7.8% by 20 minutes, indicating a small degree of retention within the vasculature of the lung. The liver count rate rose to 17.4% by 15 minutes and remained constant whereas the count rate for spleen continued to rise throughout the study.

TABLE 5(i)

CELLULAR RECOVERY FOR SIX SUBJECTS STUDIED

	Initial Neutrophil count (10^9/l)	Total Neutrophil Recovery x10^7	% Neutrophil Recovery
1	2.2	7.5	71
2	7.9	30.6	77
3	9.6	15.5	33
4	6.7	18.6	55
5	4.5	14.5	64
6	10.1	29.9	58
Mean ±SD	6.8±3.1x10^9/l	19.4±9.1x10^7	61±15%

^{111}INDIUM LABELLING OF NEUTROPHILS FOR SIX SUBJECTS

	% Labelling Efficiency	Dose (MBq)
1	63	17.8
2	85	28.2
3	78	8.2
4	75	28.7
5	63	16.0
6	76	26.2
Mean ±SD	73±8%	20.8±8.2MBq

TABLE 5(ii) PERCENTAGE OF MAXIMUM COUNT RATES FOR SIX SUBJECTS

LUNG COUNT %	(1)	(2)	(3)	(4)	(5)	(6)
12(secs)	35.1	32.3	34.7	32.4	31.5	45.5
27	20.8	20.5	30.2	16.5	15.5	31.4
55	19.1	19.4	22.9	16.2	13.4	25.8
95	18.7	18.7	17.9	14.5	13.0	21.4
150	18.6	18.5	17.2	13.2	12.7	19.1
230	19.0	17.2	15.2	11.7	12.0	17.5
315	18.8	15.7	13.0	10.6	11.3	15.3
435	17.8	13.9	10.9	9.2	10.5	13.0
585	16.7	12.2	8.9	8.3	9.8	11.1
930	12.2	10.0	7.7	7.4	9.2	9.9
1230	9.7	8.8	7.1	6.7	8.6	9.8
1529	8.0	7.8	6.9	6.1	8.3	9.6

HEART COUNT %	(1)	(2)	(3)	(4)	(5)	(6)
12(secs)	8.7	4.7	3.3	5.6	9.8	8.0
27	5.0	1.7	2.0	2.7	5.0	3.5
55	3.7	1.8	2.4	2.2	4.4	4.7
95	3.6	1.3	1.6	1.9	3.9	4.2
150	2.9	1.3	1.5	1.7	3.5	3.5
230	2.6	1.1	1.6	1.4	3.2	3.3
315	2.5	1.1	1.5	1.4	3.1	3.1
435	2.4	1.1	1.5	1.3	2.8	2.9
585	1.0	1.0	1.4	1.2	2.8	2.8
930	1.0	1.0	1.4	1.2	2.7	3.0
1230	0.9	0.9	1.5	1.2	2.7	3.1
1529	0.9	0.9	1.4	1.1	2.8	3.2

TABLE 5(iii) PERCENTAGE OF MAXIMAL COUNT RATE FOR SIX SUBJECTS

LIVER COUNT %	(1)	(2)	(3)	(4)	(5)	(6)
12(secs)	1.6	2.1	1.8	3.4	2.2	0.8
27	5.5	2.6	2.9	5.7	7.6	2.4
55	6.8	5.9	4.8	11.2	10.9	4.0
95	11.1	7.6	6.7	14.9	12.4	5.8
150	13.0	9.1	8.8	17.9	15.2	6.9
230	14.4	10.3	11.7	21.4	17.2	8.2
315	16.2	11.0	13.1	23.8	18.2	8.6
435	17.0	11.9	14.4	25.5	19.4	9.5
585	17.6	12.2	15.1	26.8	20.1	10.1
930	20.0	12.4	16.0	27.3	19.5	9.4
1230	22.0	12.2	15.8	26.9	18.6	8.6
1529	23.4	12.1	15.2	26.8	18.2	8.1

SPLEEN COUNT %	(1)	(2)	(3)	(4)	(5)	(6)
12(secs)	0.6	0.4	0.3	0.8	2.2	0.6
27	1.1	1.1	0.1	1.8	3.5	1.5
55	1.5	0.9	0.8	1.7	3.3	1.8
95	2.2	1.4	1.1	2.0	3.4	2.5
150	2.4	1.5	1.2	2.4	4.0	3.1
230	2.8	1.8	1.1	2.7	4.6	3.9
315	3.1	2.1	1.3	3.0	5.5	4.6
435	3.5	2.6	1.5	3.3	5.8	5.4
585	3.6	3.0	1.8	3.8	6.4	5.9
930	4.5	3.9	1.9	4.5	7.2	6.9
1230	4.8	4.6	2.1	4.8	8.0	7.5
1529	5.3	5.1	2.3	5.1	8.6	7.8

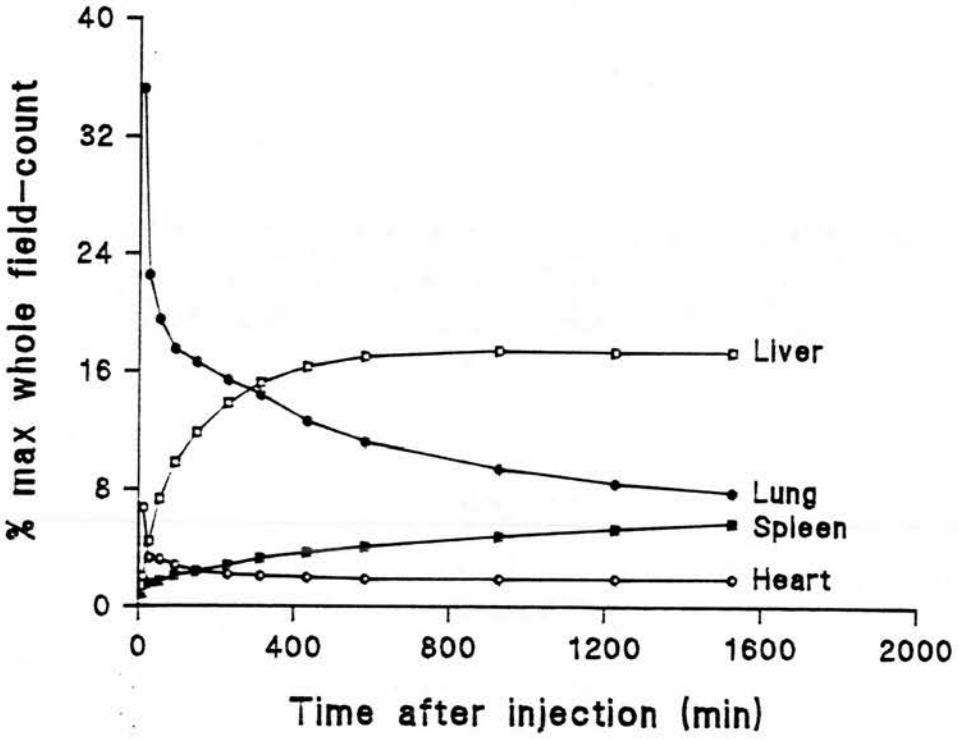
TABLE 5(iv)

MEANS OF PERCENTAGE OF THE MAXIMUM COUNT RATE FOR SIX SUBJECTS

	HEART (%)	LUNG (%)	LIVER (%)	SPLEEN (%)
secs	mean±SD	mean±SD	mean±SD	mean±SD
12	6.7±2.5	35.2±5.2	2.0±0.8	0.8±0.7
27	3.3±1.4	22.5±6.8	4.4±2.1	1.5±1.1
55	3.2±1.2	19.5±4.5	7.3±3.1	1.7±0.9
95	2.8±1.3	17.4±3.1	9.8±3.6	2.1±0.8
150	2.4±1.0	16.6±2.9	11.8±4.2	2.4±1.0
230	2.2±0.9	15.4±3.0	13.8±4.9	2.8±1.3
315	2.1±0.9	14.1±3.1	15.2±5.5	3.3±1.6
435	2.0±0.8	12.6±3.1	16.3±5.7	3.7±1.6
585	1.9±0.9	11.2±3.1	17.0±6.0	4.1±1.8
930	1.9±0.9	9.4±1.8	17.4±6.3	4.8±2.0
1230	1.9±0.9	8.4±1.3	17.3±6.6	5.3±2.2
1529	1.9±0.9	7.8±1.3	17.3±7.0	5.7±2.2

Figure 5a

Mean Indium-111 neutrophil time-activity curves



5.5 DISCUSSION

Mature neutrophils, with a 12-15um diameter, are a highly specialised, non-dividing and short-lived cell population. Canine and human neutrophils remain in the blood stream for only a short period, leaving randomly with a half-life of six or seven hours. They differentiate from a pluripotent stem cell, through stages from myeloblast to promyelocyte, myelocyte, metamyelocyte, band form and finally to the mature polymorphonuclear neutrophil leucocyte (Murphy **et al**, 1976). Only the latter two forms are found in peripheral blood and are distributed between the circulating granulocyte pool (CGL) and the marginal granulocyte pool (MGP). These together constitute the total blood granulocyte pool (TBGP). There is a dynamic equilibrium between these two pools, with the CGP constituting approximately 44% of the TBGP. This proportion, however, may increase at the expense of the MGP, in response to several stimuli including physical exercise and epinephrine administration (Athens **et al**, 1961; Muir **et al**, 1984).

Margination is a phenomenon which is thought to be exhibited throughout the capillary beds of the body. This was first demonstrated by Athens **et al**, (1961), who showed that the DF^{32}P labelled granulocytes found in the circulation 20 minutes after injection, accounted for only approximately half of the injected dose. He also showed that a further 20 to 30% of the activity could reappear in the circulation after a brisk 440 yard walk or an infusion of epinephrine. The distribution of the marginating pool in man was estimated by Peters **et al**, (1985), using a combination of dynamic gamma camera imaging and absolute quantification of ^{111}Indium tropolone labelled neutrophils in liver, spleen and blood. The marginating pool was thus calculated to represent approximately 60% of the TGBP and was distributed between spleen, liver,

lung and the remainder of the body as 35%, 25%, 10% and 30% respectively. The spleen therefore appears to be an important site of granulocyte margination. This pooling of granulocytes in the spleen normally will reach a plateau between 20 and 40 minutes and appears similar to that seen for physiologic pooling of platelets (Peters **et al**, 1985). The reversibility and the physiological nature of this splenic pooling was shown in observations that the fall in splenic activity between 40 minutes and 24 hours and 3 and 24 hours was greater in patients with inflammatory disease than in those without (Peters **et al**, 1988). Furthermore, a significant correlation was shown between this fall and the uptake of activity into inflammatory foci, indicating not only that the splenic activity was available for localisation, but that it was present in viable and functionally intact granulocytes.

Interpretation of liver activity is more difficult. It could represent any one or a composite of 3 processes: irreversible uptake (presumably damaged cells), temporary, or physiological pooling. The transit time of radiolabelled erythrocytes and neutrophils through the liver has been evaluated and found to be comparable suggesting that granulocyte activity in the liver is likely to represent a physiological phenomenon (Peters **et al**, 1985).

Physiological margination in the lung accounts for only 10% of the total marginating pool (Peters **et al**, 1985). Neutrophils activated during isolation or labelling procedures exhibit increased adhesiveness when in contact with vascular endothelium. Evidence that prolonged retention of granulocytes in the lung is due to cellular damage was provided by Thakur **et al**, (1977), who showed that heat damaged cells behaved in this way. Several patterns of cellular kinetics were described by Saverymuttu **et al**, (1983), with varying degrees of lung

hold up, dependent on the method of isolation and labelling. Good kinetics are therein described as exhibiting "rapid transit through the pulmonary vasculature". Therefore following injection, functionally viable and intact granulocytes should pass quickly through the lungs and equilibrate between the circulating granulocyte pool and the marginating pools of the liver and spleen. The assessment of in vivo behaviour of isolated cells is therefore considered to be the best indicator of cell functionality. Cells that are viable should exhibit kinetic patterns similar to that described above, while damaged cells will be "held up" in the lungs.

The behaviour of neutrophils isolated using the single step procedure (described in Chapters 2 and 3) and labelled with the gamma-emitting radioisotope, ^{111}Indium-oxine, was evaluated. The clearance of neutrophils from the lungs was rapid, with slightly greater than 80% of the total activity passing from the lungs within 90 seconds. At 20 minutes, only 7.8% of the total activity administered remained within the area of lung, indicating only a small degree of lung 'hold up'. These results correspond well with kinetic data reported in previous studies (Weiblen **et al**, 1979; Saverymuttu **et al**, 1983), showing little lung hold up and rapid pooling of neutrophils within the spleen. From these data, it can be concluded that neutrophils isolated from whole blood in Mono-Poly Resolving Medium and labelled with ^{111}Indium oxine have good in vivo kinetics and appear functionally normal.

CHAPTER 6

CLINICAL EXPERIENCE OF NEUTROPHIL ISOLATION, LABELLING AND IMAGING

6.1 INTRODUCTION

This chapter is divided into two sections. The first deals with the results of neutrophil isolation and labelling procedures performed in 100 patient studies. The second details the outcome of clinical investigation of inflammation/infection using these methods.

6.2 NEUTROPHIL ISOLATION AND LABELLING

6.2.1 Patients Studied

A total of 100 cell isolations were carried out on blood from patients with a variety of clinical conditions. They were classified here into three main groups which included those who had recently suffered acute myocardial infarction (AMI, n=58; 11F:47M; mean age 59±10 years), a group with suspected infection and/or disease of the lung (n=10; 3F:7M; mean age 66±14 years) and a group with occult infection (n=32; 12F:20M; mean age 52±18 years). Neutrophils from successful isolation procedures were radiolabelled with ^{111}Indium oxine.

Patient details and other information considered relevant were documented and included age, sex, social habits, previous medical history and a record of drug regime at the time of study.

All subjects gave informed consent.

6.3 METHODS

6.3.1 Neutrophil Isolation

Neutrophil isolation was performed on blood for 100 consecutive patients using Mono-Poly Resolving Medium (M-PRM) as described in Chapter 2.

6.3.2 Labelling with ^{111}Indium oxine

Neutrophils isolated successfully from the above patient group were radiolabelled with ^{111}Indium oxine by the method is described in Chapter 2.

6.4 CLINICAL IMAGING WITH ^{111}INDIUM LABELLED NEUTROPHILS

^{111}Indium labelled leucocytes were first used for the detection and location of suspected abscesses in man in 1977 (Thakur **et al**, 1977). Since then this method has been adopted and used successfully for detection of occult infection and a variety of inflammatory conditions including inflammatory bowel disease (Saverymuttu **et al**, 1982; Coleman **et al**, 1980; Gilbert **et al**, 1985). This technique has proved very satisfactory with specificities and sensitivities of approximately 90% reported (Peters **et al**, 1982; McDougall **et al**, 1979).

Using the method for separating neutrophils from whole blood by Mono-Poly Resolving Medium (M-PRM) and labelling discussed in this chapter, its validity in locating sites of infection and inflammation was examined.

This section of this chapter documents the clinical results for a group of patients with suspected inflammation or infection.

6.4.1 IMAGING

Planar imaging was performed, usually around 24 hours following reinjection, using a large field of view gamma camera (GEC-400T Maxicamera). The patients were positioned supine below the head of the camera. Images were acquired in the anterior, posterior and left and right oblique positions over the area or areas of interest for a fixed number of counts (usually 50,000 counts; duration of aquisition was dependent on several variables including injected dose and patient mass). An image was considered positive when indium activity was detected outwith the areas of physiological uptake observed in spleen and liver.

6.5 **RESULTS**

6.5.1 Neutrophil Isolations

A total of 100 neutrophil isolations were performed using Mono-Poly Resolving Medium. Of these, 86 were completed successfully to give an essentially 'pure' neutrophil preparation and 14 'failed'. Reasons for this were not clear initially and are discussed later.
The mean (±SD) results for initial leucocyte and neutrophils recovery are shown in Table 6(i).

In the 86 separations successfully completed an average of 49.1±17.8% of the mean initial neutrophil count of 9.3 x 10^9/l were recovered, resulting in isolation of a mean of 22.5±13.4 x 10^7 neutrophils per procedure.

A separation was considered a failure when the erythrocyte population did not settle below the layer of polymorphonuclear cells, thus making isolation impossible. Factors that may have affected this technique were examined where available and are shown in Table 6(iii).
Disturbance of red cell properties from the norm was the feature most prevalent in this relatively small group. In all cases where the erythrocyte sedimentation rate (ESR) was measured (8 of the 14), results were raised markedly above the normal range of 0-15 mm/hr (Westergren Method) with a mean±SD of 50±20mm/hr. Similarly the mean cell volume (MCV) of the red cells in seven of this group were abnormally high and ranged from 96 to 105 fl indicating the macrocytic nature of the erythrocytes in these samples. Also, low haemoglobin values, indicating anaemia, was a common feature.

The failure rate was considered within the specified patient groups, details of which are shown in Table 6(iv). The highest percentage of

failures occurred in the chest/lung group with 40%, which fell to 20% in patients with occult infection and lowest (7%) for the patients with acute myocardial infarction. It was notable that ten of the fourteen patients were hypoxic at the time of study. This may explain the prevalence of failed separations in the patient group with suspected chest infection/inflammation (40% failure rate).

6.5.2 ^{111}Indium Oxine Labelling of Neutrophils

From 86 successful separations, the neutrophils from 79 of these were labelled with ^{111}Indium oxine solution. Where possible the resultant radiolabelled cells were used clinically for detection of sites of inflammation or infection.

The mean (±SD) results and range for the neutrophil separations and labelling (n=79) are shown in Table 6(ii).
The mean labelling efficiency in this group was 72.4±12.4% and resulted in a mean injected dose of 27.1±12.0 MBq. The label was stable and no loss of radioactivity occurred on washing.

6.5.3 CLINICAL IMAGING

The imaging results were considered according to two clinical groupings; those with suspected infection or inflammation associated with the lung and those with occult infection. The results of imaging according to this classification are detailed in Table 6(v).

6.5.4 Infection/Inflammation of the Lung

Neutrophils were isolated successfully from six of this small group of ten patients. The isolated cells were radiolabelled and the patients were subsequently imaged. Reasons for study included empyema, pneumonia

and general chest discomfort. All patients were receiving medication at the time of study.

Uptake of ^{111}Indium was detected in one subject. A dense area of activity was seen in the left lung in Figure 6(a) . X-ray findings showed extensive consolidation in the lower lobe of the patient's left lung. The remaining five scans were negative; four of which were later confirmed as cases of pneumonia.

6.5.5 Occult Infection

The neutrophil isolation procedure failed in six of this group of 32 patients with suspected foci of inflammation. The cells isolated from the remaining 26 patients were labelled and scans were performed 24 hours after reinjection.

Seventeen patients had negative scans, while the remainder had positive images. The diagnoses for the positive images are shown in Table 6(v). Further investigation of those with negative scans failed to confirm the existence of undetected foci of inflammation, except in one patient where a subdiagphragmatic abscess was confirmed at post mortem. An enlarged and 'grainy' appearance of spleen in this patient was noted on inspection of planar images. However as physiological uptake in spleen is normal this prompted no additional interest in this area and consequently was reported as a "negative"' scan.

In four of the patients with uptake of the radiolabelled cells, activity was focussed in the area of abdomen suggesting the existence of inflamed or infected bowel and indeed one of the scans aided diagnosis of ischaemic colitis (Bell **et al**, 1986). The planar image in the anterior postion is shown in Figure 6(b).

Evidence of the inflammatory response to infection was demonstrated

in two subjects with unexplained rigors, the cause of which were later identified as an infection at the site of an access site and Y-graft repair.

Several 'pockets' of radioactivity were shown in a patient with septicaemia, particularly in the kidneys and the pericardial sac. Finally, the combined use of single photon computed emission tomography (SPET) and 99mTc-HSA as a blood pool marker for enhance anatomical localisation identified the uptake of ^{111}Indium labelled neutrophils in one patient in the left paravertebral area of lower thorax as a mycotic aneurysm (Bell **et al**, 1987), shown in Figure 6(c) and in a second with a cerebral abscess (Figure 6(d) and 6(e)).

Table 6(i)

Mean±SD for neutrophil isolations in 86 patients.

Initial leucocyte count	($x10^9$/l)	11.6±4.8
Initial neutrophil count	($x10^9$/l)	9.3±4.5
Neutrophil recovery	($x10^7$cells)	22.6±13.3
Neutrophil recovery	(%)	49.1±17.8

Table 6(ii)

Mean±SD of neutrophil labelling procedures (n=79) using ^{111}Indium-oxine

Number of neutrophils	($x10^7$)	23.6±13.3
Total dose ^{111}Indium oxine	(MBq)	37.3±15.1
Cell associated dose	(MBq)	27.1±12.0
Labelling efficiency	(%)	72.4±12.4

Table 6(iii)

Factors thought to influence separation procedure using M-PRM.

Patient	ESR	MCV	Hb	Hypoxic	COAD
		Myocardial Infarction			
1	N/A	105*	9.6**	N/A	+
2	N/A	103*	14.5	+	+
3	N/A	87	15.1	+	+
4	53*	99*	14.4	+	+
		Lung Infection/Inflammation			
1	14*	96*	14.8	+	+
2	30*	90	12.4**	+	+
3	N/A	105*	13.1**	+	+
4	73*	92	12.3**	+	+
		Occult Infection			
1	64*	76	10.1**	-	+
2	59*	89	11.1**	-	+
3	N/A	N/A	8.0**	N/A	N/A
4	63*	97*	12.4**	+	-
5	N/A	89	13.2**	+	+
6	45*	102*	15.3	+	+

* denotes above normal range

** denotes below normal range

N/A information not available

+ or - Yes or No

Table 6(iv)

Results of neutrophil isolations in patient groups studied.

		Total	MI	Chest/Lung	Occult Infection
Number of separations	(n)	100	58	10	32
Number of successes	(n)	86	54	6	26
Number of failures	(n)	14	4	4	6
Failure rate	(%)	14%	7%	40%	20%

Table 6(v)

Results of Imaging

	Patients Studied	Negative Images	Positive Images	Confirmed Diagnosis
Chest/Lung Infection	6	5	1	Early pneumonia
Occult Infection	26	17	9	Bowel inflammation
				" "
				" "
				" "
				Infected Wound
				Cerebral Abscess
				Septicaemia
				Mycotic Aneurysm
				Infected Aneurysm

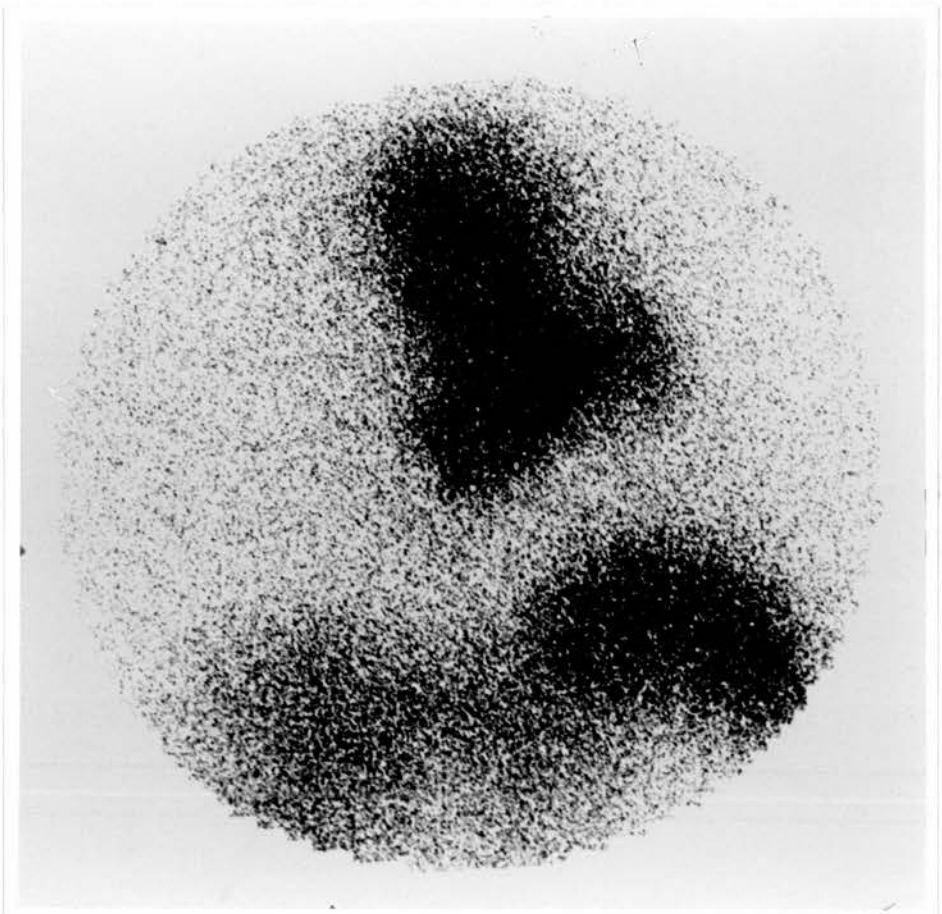


Figure 6(a)

Anterior chest image (24h) showing marked uptake of ^{111}Indium in the left upper lobe of a patient with obstructive pneumonia.

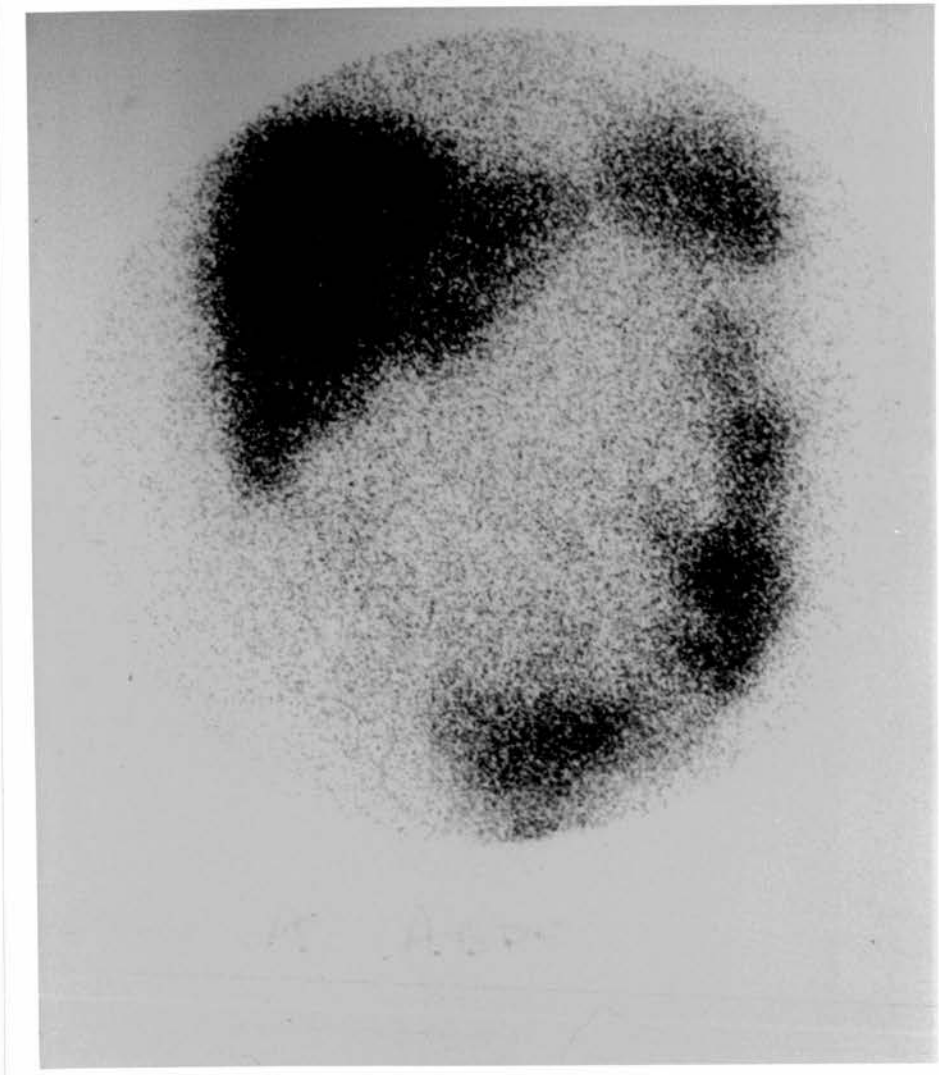


Figure 6(b)

Gamma camera image (24h) showing the anterior abdominal view from a patient with ischaemic colitis. Predominant uptake of ^{111}Indium can be seen in the sigmoid and descending colon.

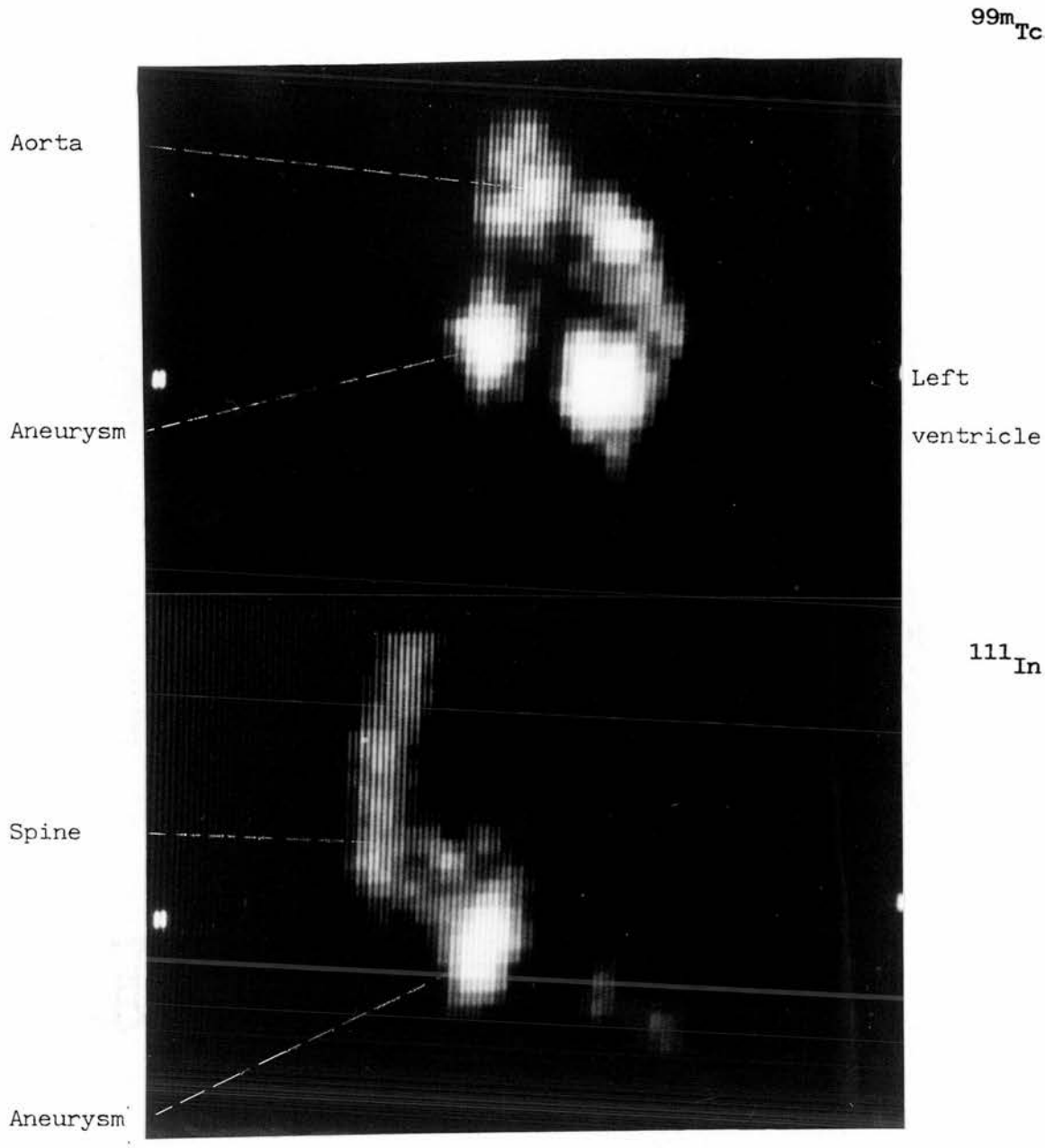


Figure 6(c)

Simultaneously acquired computer reconstruction images in the same sagittal plane.

Upper: Technetium-99m (^{99m}Tc) labelled human serum albumin showing blood pool in the left ventricle, aorta and the region of the aneurysm.

Lower: Indium-111(^{111}In) labelled neutrophils showing abnormal uptake at the site of the aneurysm, with normal uptake in the spine.

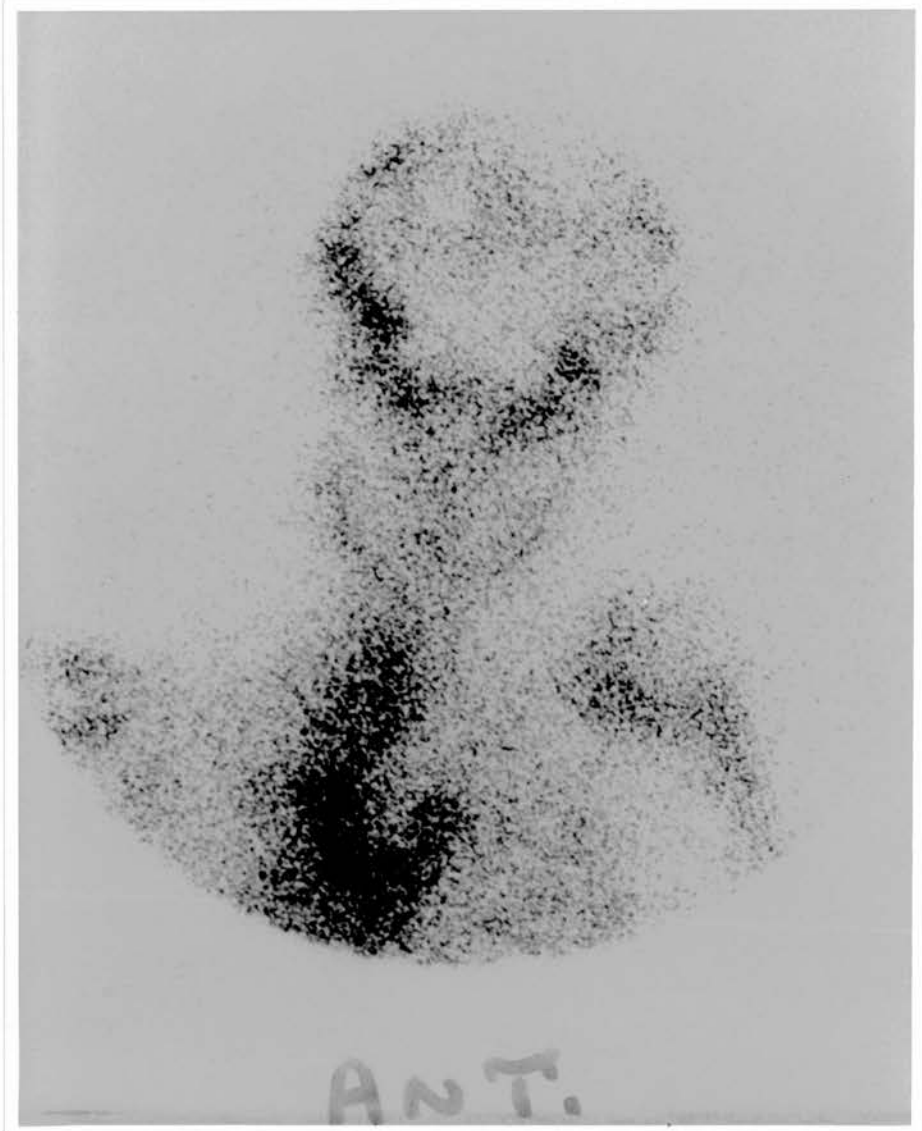


Figure 6(d)

Gamma camera image showing the anterior view from a patient with a right frontal cerebral abscess

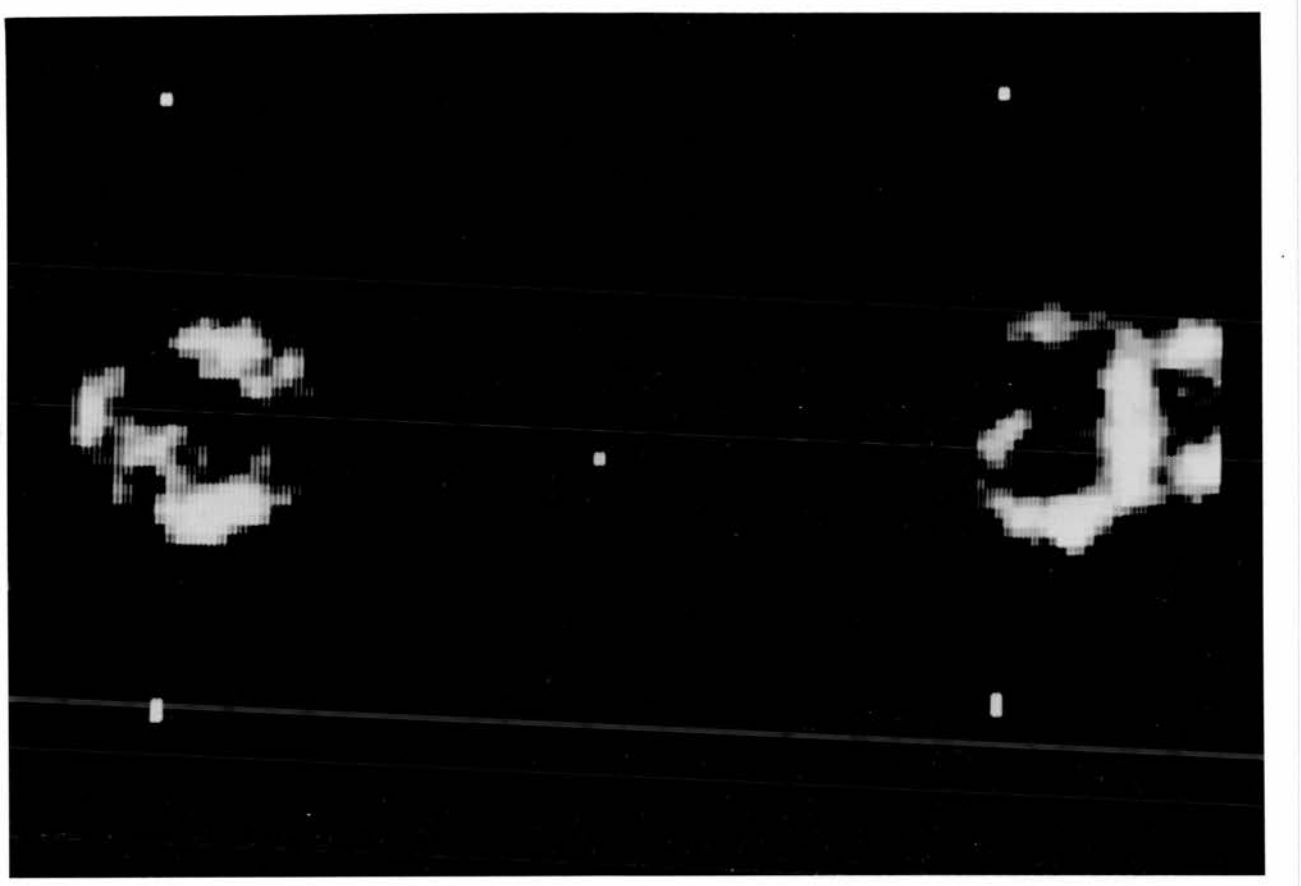


Figure 6(e)

Reconstruction ^{111}Indium transverse and coronal head images at 24h obtained using single photon emission computerised tomography (SPET) in a patient with a right frontal cerebral abscess.

6.6 DISCUSSION

Leucocytes labelled with a variety of radionuclides have been used to locate infection or inflammation since the early 1970s (Coleman **et al**, 1980; McDougall **et al**, 1979). In recent years, ^{111}Indium chelates have been used to label leucocytes for routine diagnostic purposes. Due to the non-specific nature of this isotope, the cell of interest must be isolated from the other blood cell types. Here, experience of neutrophil isolation, labelling and subsequent patient imaging is described.

In this group of 100 consecutive patient studies only 14 of the leucocyte isolation procedures were technical failures. Reasons for this were examined retrospectively and appear to relate to departure from normal erythrocyte properties. An increased ESR, MCV and reduced haemoglobin content of the red cells from these blood samples was common. Reinhart **et al**, (1989), suggested that the shape of the red cell is important in determining its sedimentation properties. He demonstrated that blood comprised of erythrocytes with irregular plasma membranes caused a reduction of the ESR.

The use of M-PRM for isolation of neutrophils in patients with anaemia, particularly in those with a microcytic, hypochromic anaemia, is discouraged by the manufacturer. Also several drugs have been demonstated to interfere with leucocyte separations. Drug regime at the time of study was also taken into account, however there was no prevalence of a particular drug or drug type in these cases. It was notable that ten of the group were hypoxic at the time of study which may lead to alteration of erythrocyte properties.

Each of these examples may alter cell shape and mass and therefore may interfere with cell migration and thus the outcome of the isolation procedure.

In the remaining 86 cases, good cell recovery was achieved quickly and easily using this method. The high purity and viability of the resultant isolate with little or no erythrocyte contamination was assessed and confirmed in Chapter 2.

In a proportion of the patients, the cell isolate was radiolabelled with ^{111}Indium oxine and suspected sites of inflammation or infection investigated using gamma camera imaging techniques. Labelling efficiency was excellent and compared well with other data published for ^{111}Indium oxine (Mountford **et al**, 1985; Thakur **et al,** 1977). Further confirmation that the labelled cells were functional was provided by the positive scans obtained in a number of the studies.

As infection is only a clinical suspicion, many of the patients referred for imaging may not have had an infective process and therefore may lead to low sensitivity and specificity of the technique. Clinical follow up in this study showed only one marked failure of the technique (ie. the patient with the subdiaphragmatic abscess). However as the labelled neutrophils normally sequestre in the spleen and liver, diagnostic accuracy in this area is limited. Positive uptake was demonstrated in only one of the cases of pneumonia, and was the only case in which cell labelling occurred early in the disease process; the importance of which will be developed in the following chapter.

The detection of activity in the bowel in four of the patient studies highlights the suitability of this method for detecting inflammation in this area. The clinical use of ^{67}Gallium citrate for this purpose has been superceded by labelled leucocytes for a number of reasons. One of the main problems is that under normal conditions ^{67}Ga citrate is excreted into the bowel and hence can give rise to false positive

results (Caffee **et al**, 1977). Also, although both are established as effective methods for abscess detection, ^{111}Indium labelled leucocytes allows distinction to be made between this and inflammation of the bowel.

No false positives or negatives were acquired in the course of these imaging studies other than the single false negative result already mentioned.

In two of the patients, this technique allowed diagnoses of intrathoracic mycotic aneurysm and ischaemic colitis with perforated bowel where routine diagnostic radiology had failed. Cases such as these demonstrate the benefits of combining radioisotopic and routine radiological imaging to aid diagnosis in difficult clinical situations.

The search for easier and more effective non-invasive methods for the detection of infection and inflammation will undoubtedly continue, however, this study confirms that this method of isolating and labelling neutrophils provides viable cells that actively infiltrate sites of inflammation.

CHAPTER 7

IMAGING THE ACUTE INFLAMMATORY RESPONSE TO MYOCARDIAL INFARCTION

7.1 INTRODUCTION

The migration of neutrophils into recently infarcted myocardium represents the initial phase of a process that leads to the removal and subsequent reorganisation of damaged tissue (Mallory **et al**, 1939, Lautsch **et al**, 1979). Neutrophil infiltration into an area of the irreversibly damaged myocardium, facilitates the breakdown of the necrotic tissue by phagocytosis. After removal of the tissue debris, capillaries and fibroblasts invade the area of infarcted myocardium, leading to the formation of collagen-rich scar tissue which eventually replaces the necrotic area (Lautsch **et al**, 1979).

These events have been well characterised histopathologically in animal studies, particularly in experimental myocardial infarction in dogs. Karsner demonstrated that the infiltration of polymorphonuclear leucocytes began as early as 12 hours after coronary occlusion, increased substantially within 24 hours and constituted a marginating zone defining the periphery of an infarct within 5 days. The polymorphonuclear and lymphocytic infiltration persisted as long as 18 days, but was invariably absent after 61 days (Karsner **et al**, 1916).

In the classic pathological study of infarction in man, the polymorphonuclear leucocyte infiltrate was initially demonstrable within 24 hours of the infarct, with the degree of penetration progressing gradually, reaching its peak at around four days. Necrotic changes in the neutrophils themselves were evident by the fifth and sixth days and by day fourteen had practically disappeared (Mallory **et al**, 1939).

During the initial phase of the inflammatory response, neutrophils

undergo a complex series of biochemical changes facilitating the release of oxygen-derived free radicals and proteolytic enzymes which promote tissue lysis (Sbarra **et al**, 1959). Although these processes are important for the control of bacterial infection, it is also possible that they may lead to the destruction of the surrounding potentially viable tissue.

The large accumulation of neutrophils and their subsequent release of oxygen derived free radicals and proteolytic enzymes might lead to the extension of myocardial damage after myocardial infarction. Several studies in animals have demonstrated that neutrophil inhibition results in the reduction in size of the experimentally induced infarct (Romson **et al**, 1982; Thakur **et al**, 1979).

Very few studies of this nature have been undertaken in man, however a study by Davies and colleagues attempted to image the acute inflammatory response to myocardial infarction in a group of 36 patients with ^{111}Indium labelled neutrophils. ^{111}Indium activity in the myocardium, representing neutrophil uptake, was demonstrated in 21 of the subjects. The outcome of imaging was found to be influenced by the time to reinjection of labelled cells after chest pain and patient age, but not by the site of infarct, peak serum creatine kinase, peripheral leucocyte count, cell labelling efficiency or leucocyte function (Davis **et al**, 1981)

Using the methods developed for isolating a 'pure' neutrophil preparation and labelling with ^{111}Indium oxine, we attempted to image the acute inflammatory response to myocardial infarction in a group of 30 patients. Factors influencing the outcome of imaging were also assessed.

7.2 PATIENT GROUP

Thirty patients who had recently experienced acute myocardial infarction detailed in Chapter 6 were studied. The patients were selected on the basis of the diagnosis of acute myocardial infarction (AMI) based on a history of prolonged ischaemic pain lasting longer than thirty minutes, ECG changes associated with AMI and a rise in creatine kinase to at least twice the upper limit of normal.

Details of patient age, sex, site of infarction, peak creatine kinase and time from initial onset of chest pain to reinjection of autologous ^{111}Indium labelled neutrophils are shown in Table 7(i).

All patients gave informed consent and the study had the approval of the Institute's Ethical Committee.

7.3 MATERIALS AND METHODS

7.3.1 Neutrophil Isolation, Labelling and Reinjection

Venous blood (60ml), was taken from each patient within hours of experiencing AMI. The time of sampling from the 'major onset' of chest pain was noted along with a note of any medication the patient was receiving at that time.

Neutrophils were isolated from this volume of blood and labelled with ^{111}Indium oxine using the methods described in Chapter 2.

The autologous ^{111}Indium labelled neutrophils were reinjected into a fresh site in the patients' arm.

Cell isolation and radiolabelling were completed within two hours of venesection in all thirty patients.

The protocol is shown in diagramatic form in Figure 7(a).

7.3.2 Human Serum Albumin with ^{99m}Technetium

A Blood Pool Marker

In 12 of the 24 patients who had single photon emission computed tomography (SPET) performed, blood pool was imaged using ^{99m}Technetium labelled human serum albumin (^{99m}Tc-HSA), to further improve anatomical localisation.

^{99m}Tc-HSA was prepared from a freeze-dried kit (TCK-2, CIS(UK) Ltd.) and injected 10 minutes prior to imaging with SPET.

7.3.3 PLANAR IMAGING

Imaging was performed 24 hours post re-injection on all 30 patients. This time was chosen as it was shown to be the optimal time for scanning patients who have received ^{111}Indium labelled neutrophils (Thakur **et al**, 1979). Moreover, by 24 hours, sufficient time has elapsed for equilibration of the granulocyte pool to have occurred, thus minimising false positive results. Consequently, all patients were scanned as close to 24 hours post-injection as hospital routine would allow.

Patients were imaged supine under a large field of view gamma camera (GEC-400T Maxicamera) interfaced to a PDP11-34 Computer (Digital Equipment Corporation). Planar images were acquired in the anterior, left anterior oblique and the left lateral positions. The acquisition period was set to accumulate 100 000 counts in the field of view.

7.3.4 SPET (Single Photon Emission Computed Tomography)

In 24 of the 30 patients, SPET was also performed in an effort to improve anatomical definition. SPET was not felt appropriate in all cases as it increased the imaging time. Patients considered to be 'unwell' were not subjected to this test.

To further improve organ localisation ^{99m}Tc labelled human serum albumin (^{99m}Tc-HSA) was injected ten minutes prior to imaging, to allow visualisation of blood pool relative to the heart.

'Windows' were set at 171-281 KeV and 128-281 KeV thus allowing simultaneous acquisition of indium and technetium activity. A sequence of 64 of these images were acquired by rotating the camera head through 180 degrees at a fixed distance from the patient, starting at the right anterior oblique position (RAO). The patient remained perfectly still throughout the whole of the procedure, which took approximately 32 minutes. On completion, the images were reconstructed using software written locally for the PDP11/23+.

7.3.5 STATISTICAL ANALYSIS

Data from the groups were compared using unpaired t-test, exact probability or unpaired Wilcoxon rank sum as appropriate. Values of $p<0.05$ were considered not significant.

7.3.6 IMAGE ANALYSIS AND INTERPRETATION

An independant observer, who was unaware of the patient identity or clinical history was asked to grade the planar and SPET images as positive, (where ^{111}Indium activity was clearly defined in the region of the heart) and negative (where there was no detectable uptake ^{111}Indium activity in the myocardium).

7.4 **RESULTS**

7.4.1 PLANAR AND SPET IMAGING

The results of planar and SPET imaging are shown in Table 7(ii). The planar images were judged positive or negative as described above. Planar imaging was performed on all 30 patients. Twentyfour of the 30 patients also underwent SPET to allow improved anatomical localisation. Where SPET was available, the outcome of imaging agreed with that of the planar images. In deciding the outcome of imaging of those patients with negative planar scans, SPET was also taken into account. As a result, six patients showing no indium activity in the planar images, had positive uptake in the area of the heart on the SPET reconstructed images.

In 23 of 30 patients studied, uptake of indium labelled neutrophils in the area of infarcted myocardium was detected by either planar images alone or by a combination of planar imaging and SPET.
Three patterns of uptake were seen and were classified as focal myocardial uptake (11 patients), diffuse myocardial uptake (2 patients) and a 'doughnut' shaped uptake (3 patients), see Figure 7(c). One patient showed both diffuse and focal uptake in the myocardium and is shown in Figure 7(b). Of the six patients in whom the planar images were considered negative, SPET reconstruction showed localised uptake within the myocardium, Figure 7(d). For the remaining seven patients no uptake of indium activity was detectable either on planar or SPET images.

In addition, in 12 of the patients who had SPET performed, imaging of blood pool was permitted by imaging ^{99m}Technetium activity. The mean$\pm$SD injected dose was 44.7$\pm$5.7 MBq. Where necessary the ^{99m}Tc images were scrutinised together with that for indium uptake to allow differential

localisation of tissue and blood pool-associated activity.

7.4.2 INFLUENCING FACTORS

The patients were grouped according to the outcome of imaging (ie. positive n=23; or negative n=7) and factors which may have influenced the results were documented. (See Table 7(iii))
Patient age, sex, site of (inferior; INF or anterior; ANT) and size of infarct, peripheral leucocyte count, injected neutrophil number and radioactivity dose, as well as the time from onset of chest pain to reinjection, were compared between the two groups.

There was no statistical difference in age (62.0±10.8 years vs 59.8±9.4 years) or sex distribution (6F:17M vs 2F:5M) in patients in whom images were positive or negative.

The site of infarct (10 INF:13 ANT vs 3 INF:4 ANT), as well as the size of infarct, as indicated by peak creatine kinase (2023.5±916.0 U/l vs 1825.0±1214.0 U/l) also did not appear to influence the uptake.

The patient peripheral leucocyte count ($12.9\pm3.2\times10^{9}$/l vs $12.5\pm3.4\times10^{9}$/l), neutrophil number (2.7×10^{8}cells vs 2.6×10^{8}cells) and activity of the radiolabelled neutrophils injected (32.8±8.4MBq vs 29.4±8.3 MBq) did not differ significantly for positive and negative studies respectively.

The interval between onset of chest pain and reinjection of autologous labelled neutrophils however, was significantly shorter for the group with positive images (20.3±6.4 hours) when compared to those whose images were negative (27.6±5.8 hours: $p<0.02$).

The patient group was then subdivided according to three time intervals; those injected within 18 hours of chest pain; between 18 and 24 hours and between 24 and 36 hours. Details of the imaging results

within these time periods are shown in Table 7(iv) and Figure 7(f).

Nine subjects of the 30 patients were reinjected within 18 hours of the onset of chest pain and all had positive images. As the interval between onset of chest pain and the time of reinjection increased, the frequency of positive images reduced with 10 of the 12 patients (83%) reinjected between 18 and 24 hours with positive images and only four of the nine of those injected between 24 and 36 hours after infarction had positive scans (46%).

TABLE 7(i)

Patient details

Number of subjects studied	30
Sex (F:M)	8:22
Location of acute myocardial infarct	13INF:17ANT
Peak creatine kinase (U/l)	1972±980
Interval from onset of chest pain to reinjection of ^{111}In neutrophils (h)	21.9±6.9
Total leucocyte count (10^9/l)	12.8±3.2
Number of neutrophils injected (10^7)	26.8±8.2
Activity of ^{111}In administered (MBq)	32.0±8.2
Activity of ^{99m}Tc administered (MBq) (n=12)	44.7±5.7

TABLE 7(ii)

Results of planar and SPET imaging for 30 patients.

		POSITIVE (+) IMAGE		NEGATIVE (-) IMAGE
Patient Numbers		Planar (+) SPET (+)	Planar (-) SPET (+)	Planar (-) SPET (-)
30		17	6	7
Total	30	23		7

TABLE 7(iv)

Results of planar and SPET imaging for 30 patients grouped according to time between onset of chest pain and reinjection.

TIME	PATIENTS	POSITIVE (+) IMAGE		NEGATIVE (-) IMAGE	% (+) IMAGE
		Planar (+) SPET (+)	Planar (-) SPET (+)	Planar (-) SPET (+)	
18h	9	7	2	0	100
18-24h	12	7	3	2	83
24-36h	9	3	1	5	44

TABLE 7(iii)

Mean(SD) for patients with positive and negative images.

	POSITIVE IMAGE		NEGATIVE IMAGE
Age (years)	62.0(10.8)	(1)	59.8(9.4)
Sex (M:F)	17M:6F	(2)	5M:2F
Site of infarct	10INF:13ANT	(2)	3INF:4ANT
Peak creatine kinase (U/l)	2023(916.)	(1)	1825(1214)
WBC ($x10^9/l$)	12.9(3.2)	(1)	12.5(3.4)
Neutrophils injected ($x10^8$)	2.7(0.9)	(1)	2.6(0.7)
^{111}Indium dose (MBq)	32.8(8.4)	(1)	29.4(8.2)
Time between onset of pain to injection (hours)	20.3(6.4)	(3) p 0.05	27.6(5.8)

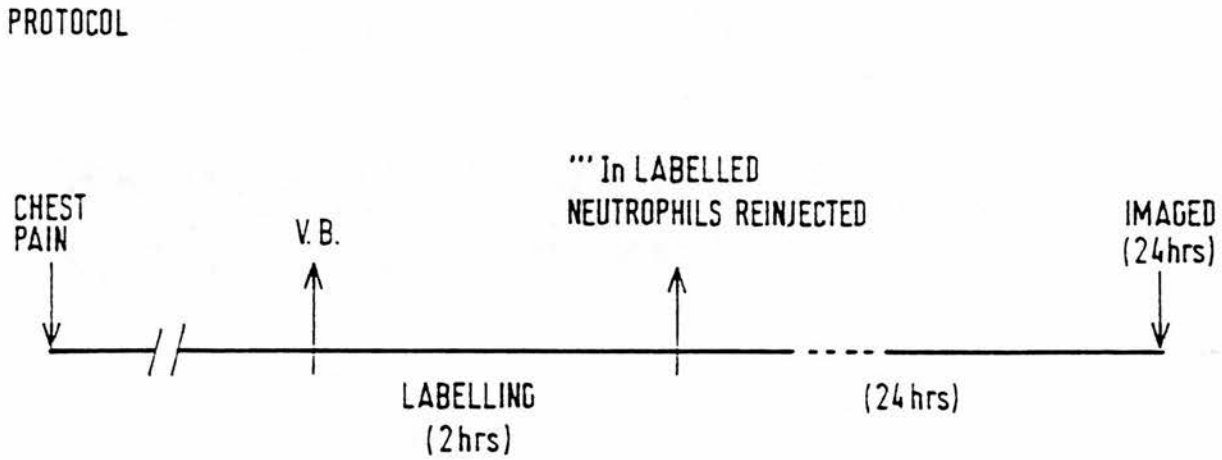
Statistical Analysis used in each case

(1) Unpaired t-test

(2) Exact probability

(3) Unpaired Wilcoxon rank sum test

Figure 7(a)


PROTOCOL
CHEST PAIN
V. B.
''' In LABELLED NEUTROPHILS REINJECTED
IMAGED (24hrs)
LABELLING (2hrs)
(24 hrs)

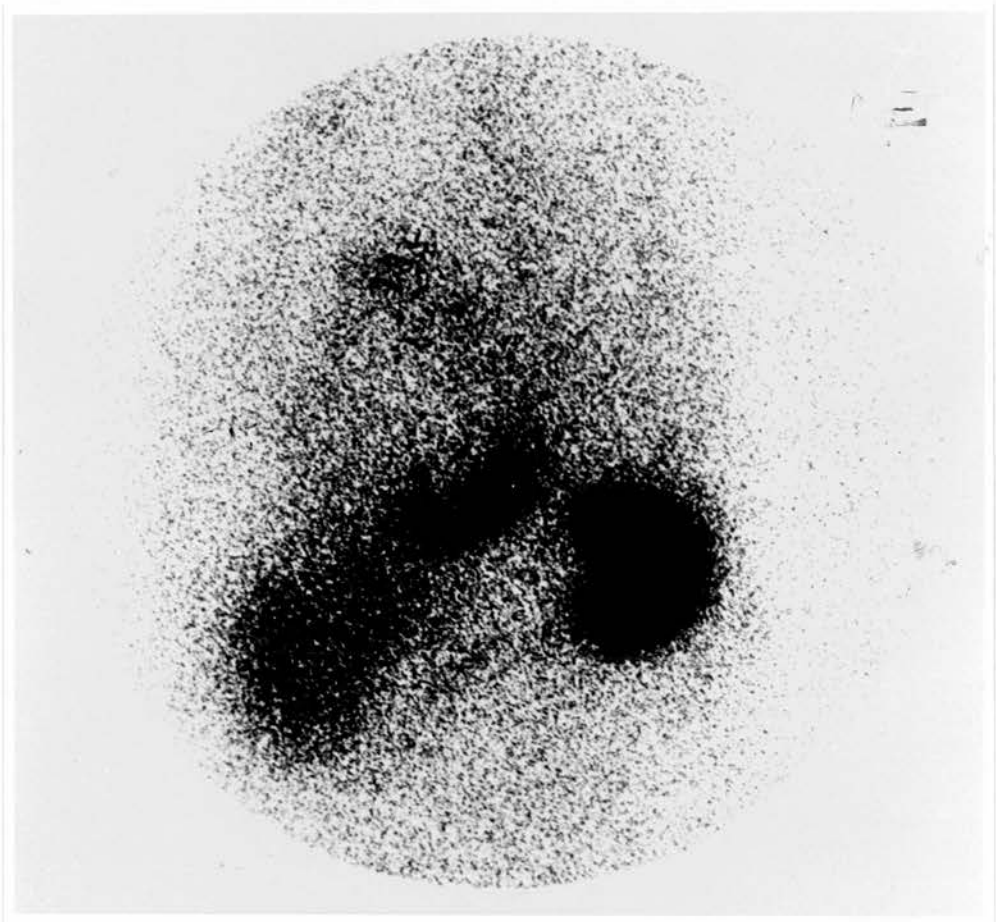


Figure 7(b)

Upper: Planar image in the left anterior oblique view showing normal uptake of ^{111}Indium-labelled neutrophils in the liver and spleen, with diffuse uptake in the region of the heart and an area of focal uptake in the inferior wall of the left ventricle.

Lower: The line drawing shows the areas of uptake.

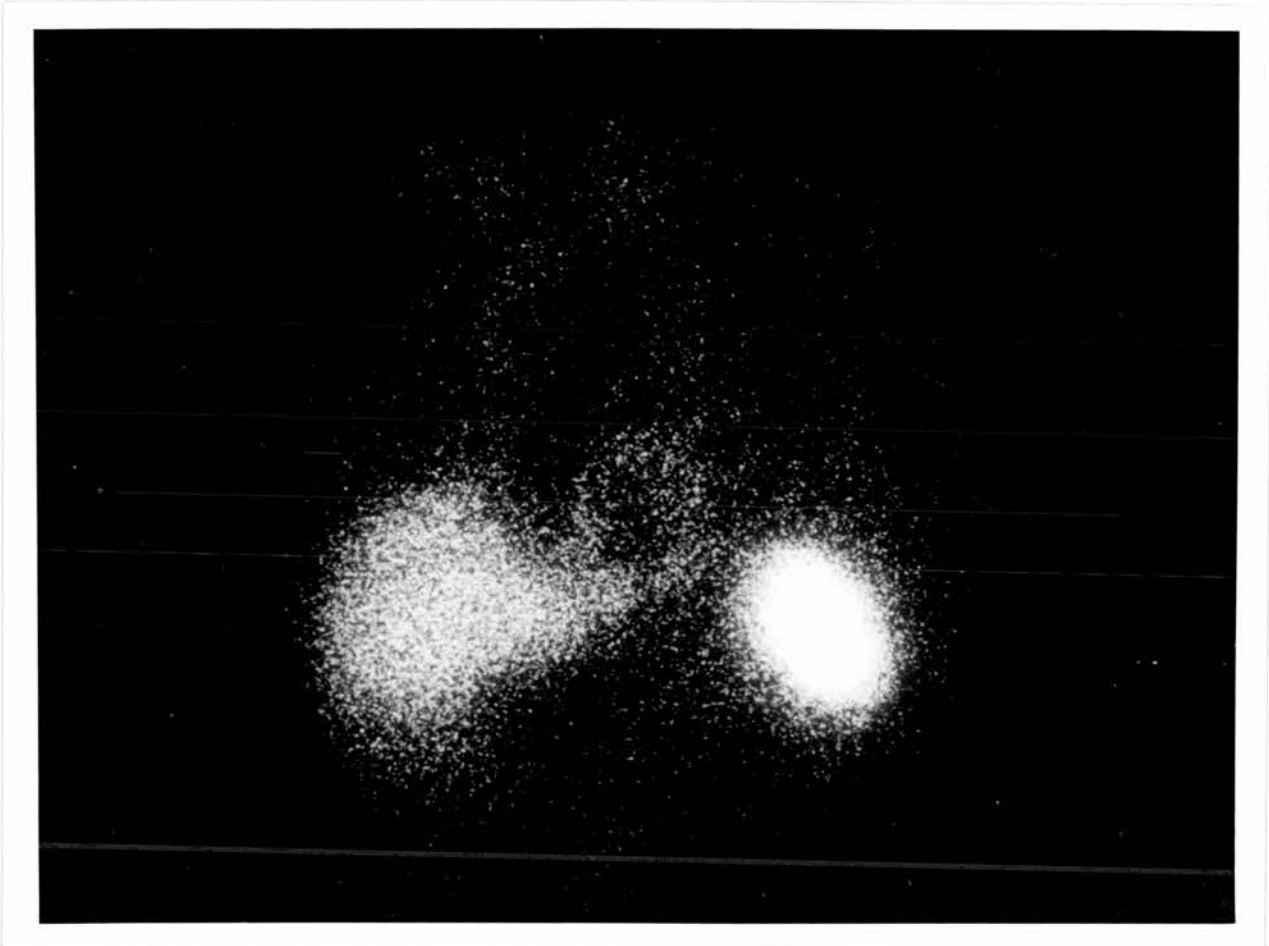
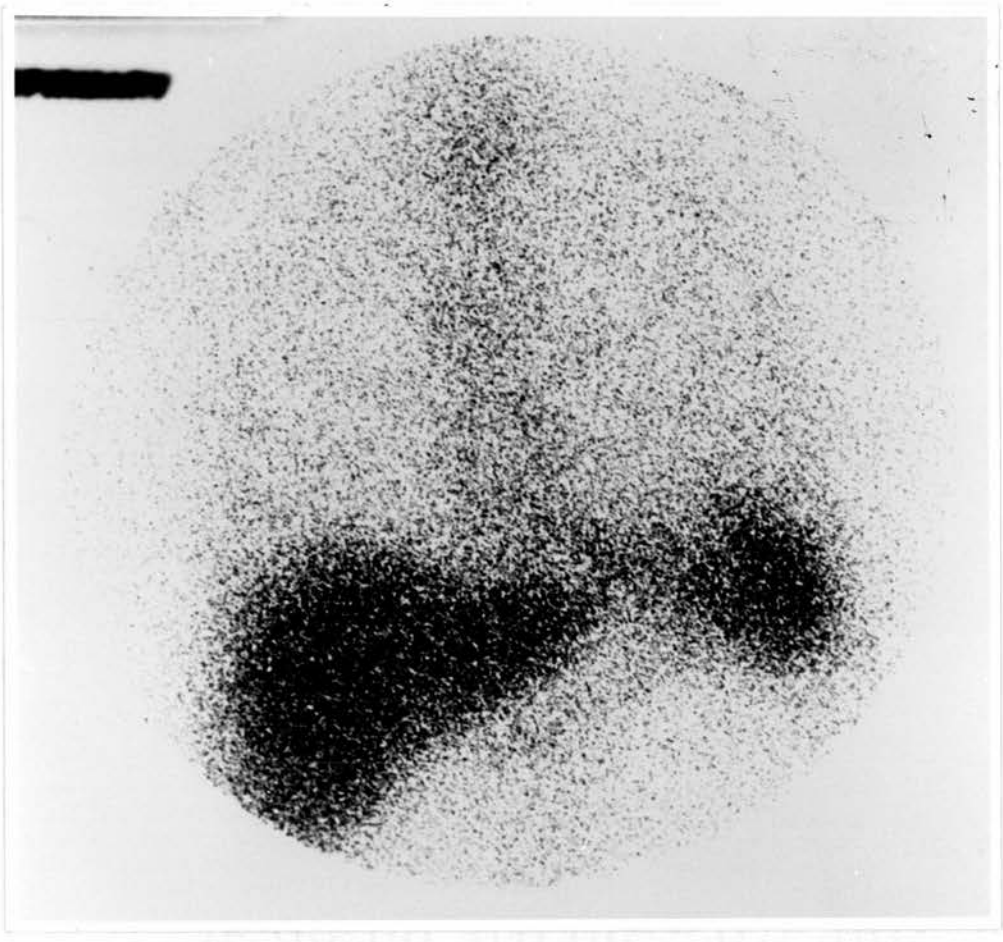


Figure 7(c)

Planar image in the left anterior oblique view showing normal uptake in spleen and liver with "doughnut" shaped activity in the area of the heart.



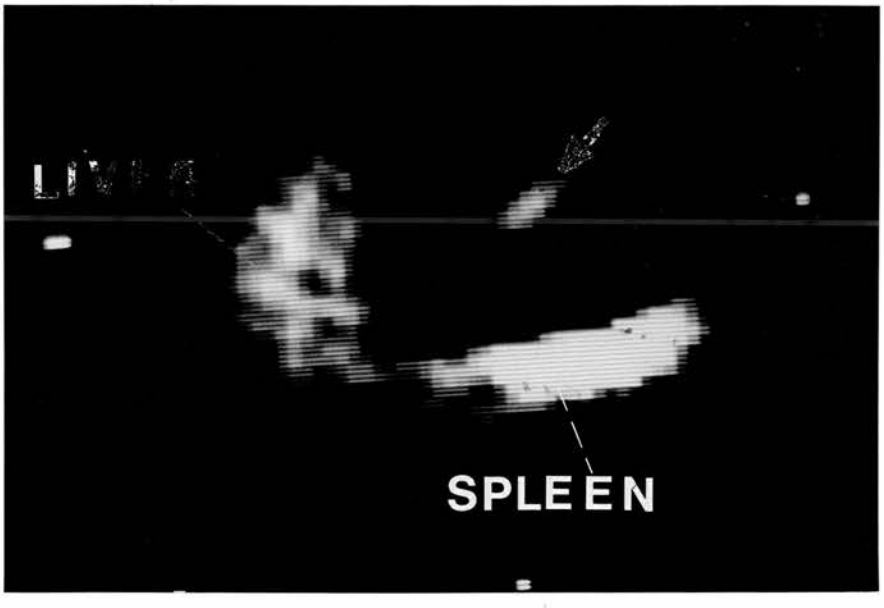


Figure 7(d)

Upper: Anterior planar image with normal uptake in liver and spleen and no definite myocardial uptake.

Lower: Single photon computed emission tomographic (SPET) image in the transverse plane showing uptake within liver and spleen and an area of focal myocardial uptake.

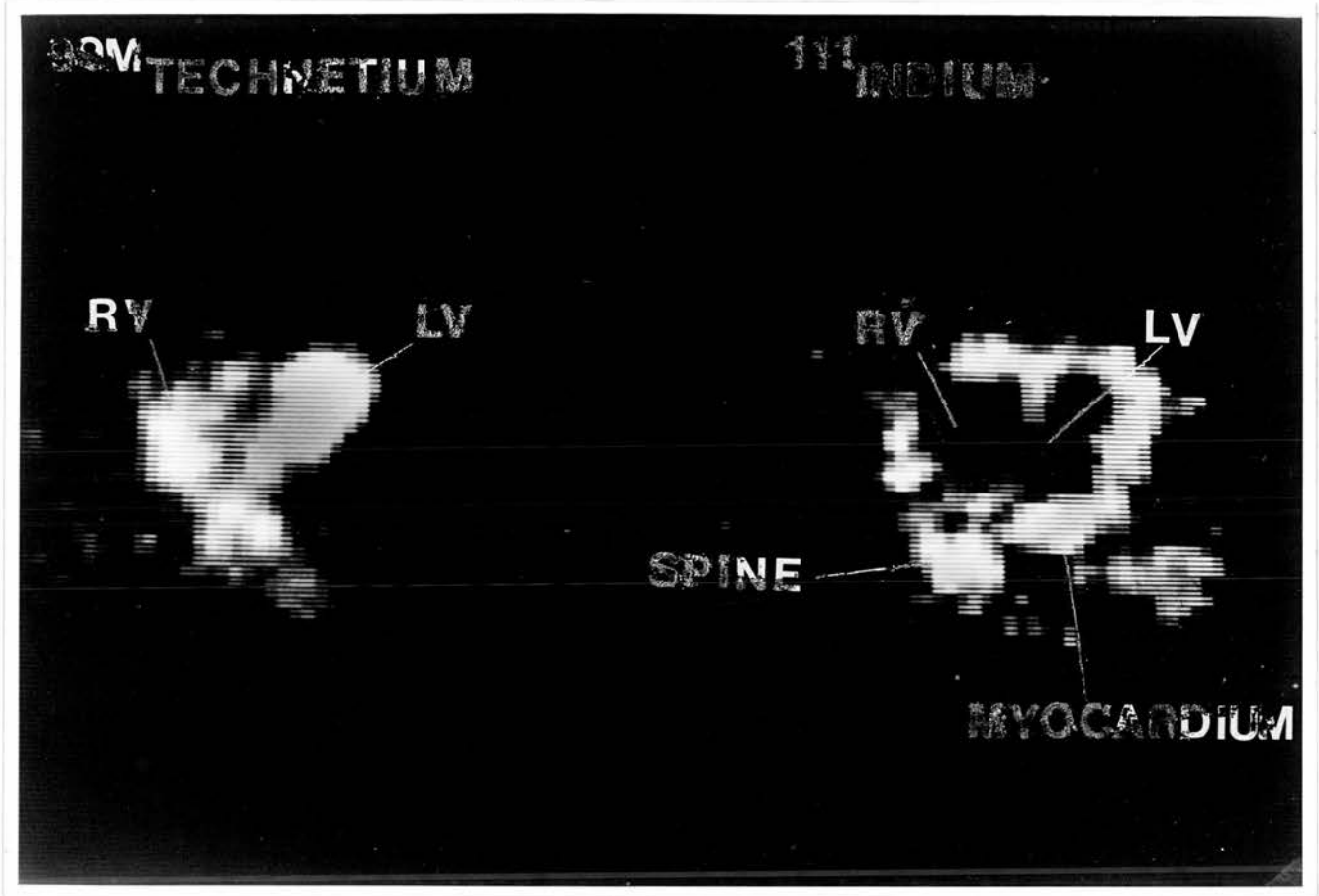
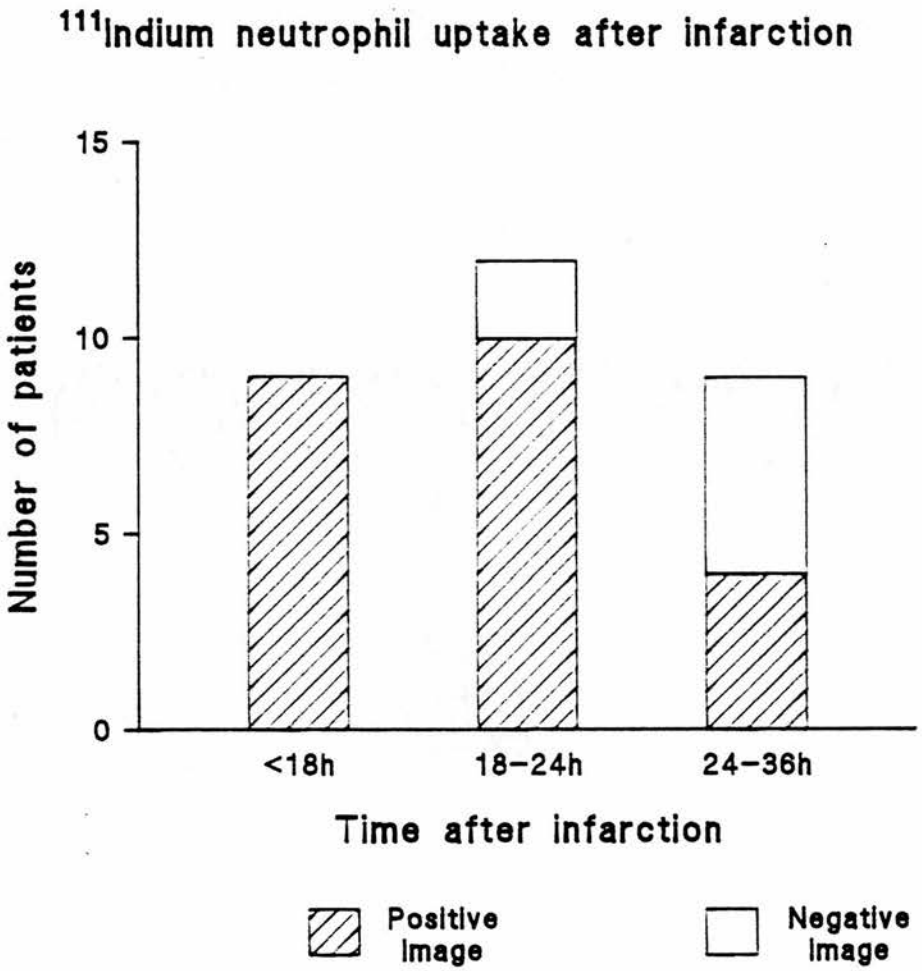


Figure 7(e)

Simultaneous SPET images in the transverse plane. The ^{99m}Tc image shows the blood pool in the left (LV) and right ventricle (RV).
The corresponding ^{111}Indium image shows extensive uptake within the myocardium of both ventricles.

Figure 7(f)


111Indium neutrophil uptake after infarction
15
10
5
0
Number of patients
<18h
18–24h
24–36h
Time after infarction
Positive Image
Negative Image

Did [illegible] or [illegible]
[illegible] influence
[illegible]?

7.5 DISCUSSION

This study shows that the acute inflammatory response to myocardial infarction in man can be imaged using autologous ^{111}Indium labelled neutrophils.

When the patients were divided according to the time from onset of chest pain to the injection of labelled cells, it was found that the highest frequency of positive images were obtained for those patients reinjected within 18 hours of the major onset of chest pain (77% of patients). This high incidence of positive results fell as the interval from the estimated time of infarction increased and was lowest in the patient group in which the time interval was furthest from the event (24-36 hours), with only 44% of the patients with positive scans.

The high incidence of positive images (77%) obtained here compares favourably with that of Davies (58%), (Davies **et al**, 1981). This may in part be due to the earlier reinjection of the labelled cells after onset of chest pain; 8-36 hours in our study compared with 18-112 hours in the study of Davies and colleagues (Davies **et al**, 1981).

None of the other factors investigated influenced the outcome of imaging, and again contrasted with the findings from the study by Davies **et al**, (1981), who found that the age of the subject was also a determinant of the outcome of imaging.

The use of planar imaging makes it difficult to determine the site of the activity. Here the additional use of single photon emmission computed tomography (SPET) increased the number of positive images by allowing spatial separation of positive myocardial uptake of indium from adjacent bone, liver and spleen. Also dual isotope SPET with ^{99m}Tc-HSA allowed unequivocal differentiation between blood pool and ^{111}Indium activity localised within the myocardium.

The temporal relationship found here, suggests that the stimulus for activation and migration of the neutrophil population into the site of the myocardial infarct appears to be early and somewhat transient.

Whilst histologic studies demonstrate the number of leucocytes that have accumulated in the tissue since the onset of infarction they do not reflect the temporal sequence or the rate of this process. In contrast cardiac imaging 24 hours after the injection of cells reflects only ^{111}Indium neutrophil infiltration that occurred during that time.

Although imaging indium-labelled autologous neutrophils in patients with acute myocardial infarction allowed the acute inflammatory response to myocardial damage to be imaged, this technique should not be considered as a method of diagnosis or localisation of acute myocardial infarction as existing technology is more appropriate. It may however provide a useful means of monitoring the effects of therapy aimed at improving the prognosis of these patients.

Several methods aimed at reducing infarct size thus improving patient prognosis have received interest. Attention has focussed on the use of thrombolytic therapy to achieve these objectives (Mathey **et al**, 1981; Been **et al**, 1985). Administration of thrombolytic agents such as streptokinase and tissue plasminogen activator, can produce coronary reperfusion and therefore may improve myocardial salvage. However it is thought that the sudden reintroduction of oxygen and neutrophils into ischaemic tissue on reperfusion may also produce a chain of events leading to further tissue necrosis, a situation aptly described by Braunwald as "the sword of Damocles" (Braunwald **et al**, 1985).

Therefore if these and other therapies do exert their action by inhibiting neutrophil migration into myocardium, then this method should allow the extent and temporal nature of neutrophil uptake to be

monitored.

CHAPTER 8

WHITE CELL COUNT, NEUTROPHIL ACTIVATION AND FREE RADICAL ACTIVITY IN PATIENTS WITH STABLE ISCHAEMIC HEART DISEASE AND ACUTE MYOCARDIAL INFARCTION

8.1 INTRODUCTION

Following acute myocardial infarction, myocyte damage occurs, initiating an acute inflammatory response. This inflammatory response was generally viewed as a secondary process serving the purpose of demolition and restoration of dead and dying tissue, however recent work raises the possibility that the influx of neutrophils during this response may contribute to the extension of myocardial cell death.

The inflammatory response is characterised by migration of neutrophils into the infarcted myocardium, which in animal (Sommers **et al**, 1964) and human studies (Mallory **et al**, 1939) has been shown to occur within 24 hours with a maximal response by 96 hours.

Neutrophils, once activated, are capabable of liberating proteolytic enzymes and several reactive oxygen-derived free radicals, which are potentially harmful to the surrounding tissue if poorly regulated. Neutrophil elastase, a serine protease contained in the azurophilic granules of the neutrophil is among the substances released and is an established marker of neutrophil activation (Plow, 1982).

Reactive oxygen free radical species released during activation of neutrophils can also cause cellular damage, lysis and disruption of endothelium (Harlan, 1985). In vivo assessment of free radical activity is difficult, due to their highly reactive and short-lived nature. However these species once produced, interact with adjacent molecules, particularly polyunsaturated fatty acids (Halliwell **et al**, 1984). The

diene conjugated non-peroxide isomer of linoleic acid (PL-9,11-LA') has been used as a marker of free radical activity (Iverson **et al**, 1985).

Here these markers of neutrophil activation and free radical activity were measured in a group of patients with acute myocardial infarction, stable ischaemic heart disease and a control group of healthy volunteers.

8.2 METHODS

8.2.1 Subjects

Three groups were studied. A group of 20 patients (15M:5F; mean age 62 years) with a diagnosis of acute myocardial infarction based on a history of prolonged ischaemic chest pain (duration longer than 30 minutes), electrocardiographic changes associated with myocardial infarction and a rise in creatine kinase at least twice the upper limit of normal. Thirty patients (24M:6F; mean age 62 years) with stable angina and documented ischaemic heart disease (previous myocardial infarction or coronary angiography) attending the out-patient clinic. The normal control group comprised of 35 healthy volunteers from hospital staff (31M:4F; mean age 31 years). All gave informed consent and the study had the approval of the Institute's Ethical Committee. Subject details are given in Table 8(i).

8.2.2 Blood Sampling

In patients with myocardial infarction, venous blood was taken for estimation of full blood count, creatine kinase, plasma neutrophil elastase (PNE) and the diene conjugated non-peroxide isomer of linoleic acid (PL-9,11-LA'). Samples were taken as close to the time of admission

as possible and thereafter every 6-8 hours over the remainder of the 48 hour period. Normal volunteers and out-patients with ischaemic heart disease had blood taken for full blood count, neutrophil elastase and PL-9,11-LA'. Samples for neutrophil elastase and PL-9,11-LA' estimation were separated and stored at -20°C until assayed, as described in Chapter 2, within a week of sampling. Full blood counts were performed on a sequestrene sample, using a Sysmex E5000 Toa Electronics Ltd, Kobe, Japan. Creatine kinase in heparinised plasma was measured by the hospital clinical chemistry department.

8.2.3 STATISTICS

The Kolmogarov-Smirnov test showed that the data was not normally distributed. Results are therefore expressed as the median and range. The data was analysed non-parametrically using the Mann-Whitney test for two independent samples. Values of $p<0.05$ were taken as significant.

8.3 **RESULTS**

8.3.1 White Cell Count (WBC)

The white cell count was significantly higher in patients with ischaemic heart disease (6.7×10^9/l, 5.2-12.6, $p<0.01$) than in the normal group (5.8×10^9/l, 3.4-9.3).

The peak white cell counts for the period of study in patients with acute myocardial infarction (13.2×10^9/l, 8.8-19.4) were significantly greater than isolated measurements for both the normal controls ($p<0.001$) and those patients with stable ischaemic heart disease ($p<0.001$). See Figure 8(a). The peak value occurred early after infarction (15 ± 10h) and fell over the remaining period of the study.

8.3.2 Plasma Neutrophil Elastase (PNE)

The concentration of plasma neutrophil elastase was significantly lower in controls (18.6ng/ml, 9.2-51.0) than in patients with chronic ischaemic heart disease (25.8ng/ml, 12.2-49.5; $p<0.05$). Peak plasma neutrophil elastase for the patients with myocardial infarction (61.0ng/ml, 16.2.0-128.0) was significantly greater than both the ischaemic group ($p<0.001$) and the normal control group ($p<0.001$). See Figure 8(b).

8.3.3 PL-9,11-LA'

There was no significant difference in PL-9,11-LA' between normal volunteers (19.3umol/l, 7.5-32.9) and patients with ischaemic heart disease (19.8umol/l, 7.9-43.2).

Peak levels of PL-9,11-LA' in patients with acute myocardial infarction were 30.6umol/l, (11.5-57.3) and were raised significantly over that for the normal group ($p<0.001$) and the ischaemic patient group ($p<0.001$). See Figure 8(c).

8.3.4 Temporal Relationship

In the group of patients with myocardial infarction the time at which the plasma levels of neutrophil elastase and PL-9,11-LA' peaked, differed. The peak level of PNE occurred at around 25 hours (4-49 hours) while the PL-9,11-LA' level occurred earlier at 12 hours (1.5-29 hours).

8.3.5 Influencing Factors

Neither plasma neutrophil elastase nor PL-9,11-LA' correlated with subject age or peripheral leucocyte count in the normal controls and patients with acute myocardial infarction. There was however a weak

correlation of PL-9,11-LA' with WBC ($r=0.47$; $p<0.01$), but not PNE, in the patients with stable ischaemic heart disease.

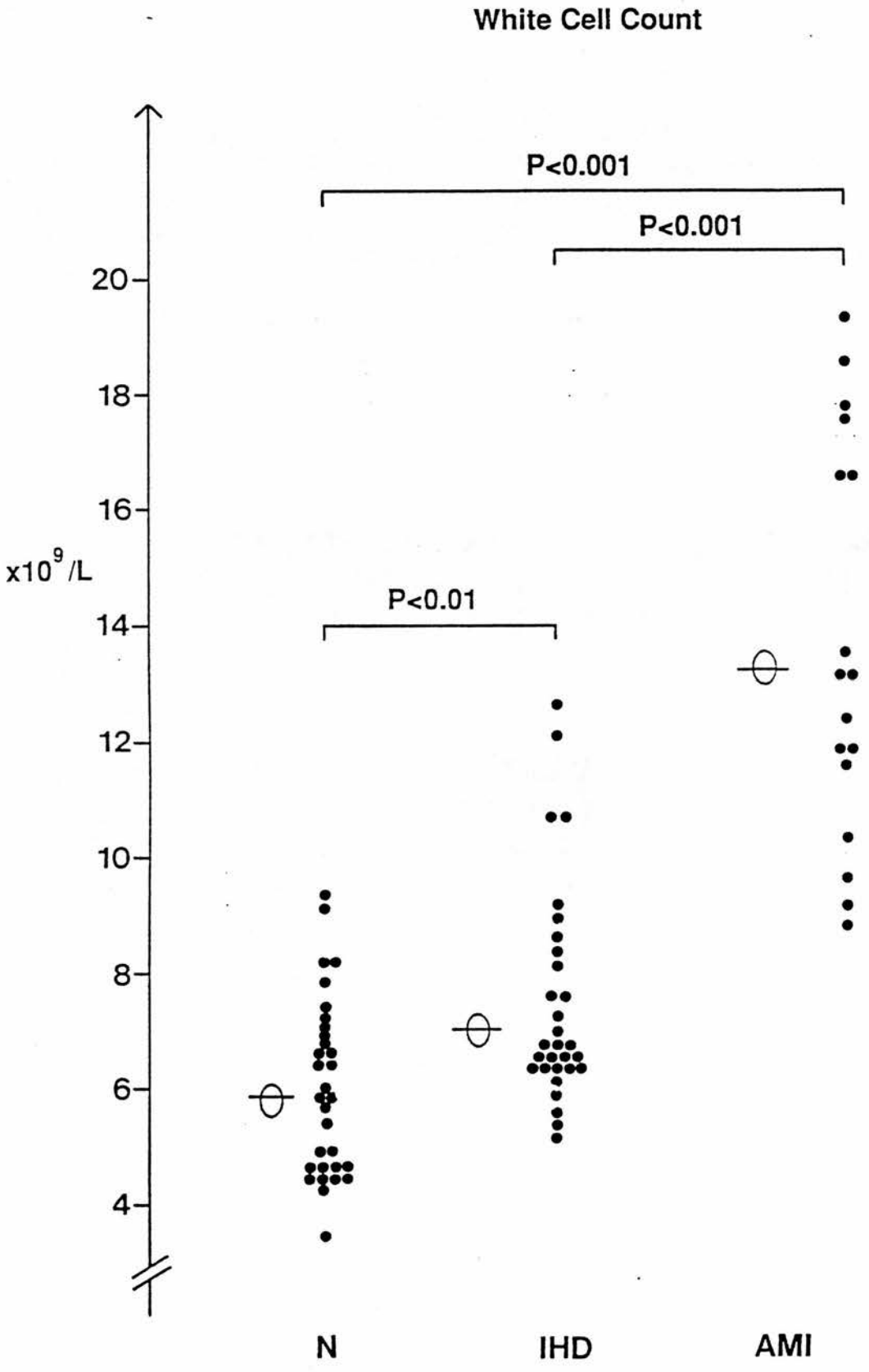
In the group with acute myocardial infarction there was a weak correlation of peak creatine kinase with PL-9,11-LA' ($r=0.47$; $p<0.05$), but there was no correlation with PNE.

Table 8(i)

Details of patients with acute myocardial infarction (AMI, n=20), stable ischaemic heart disease (IHD, n=30) and a group of normal controls (N, n=35). Median and ranges are shown where appropriate.

	Age (years)	Sex M:F	Infarct Site ANT:INF	Creatine Kinase (U/l)
N	31 (22-63)	31:4	-	-
IHD	62 (37-76)	24:6	-	-
AMI	62 (35-74)	15:5	11:9	1703 (624-3190)

Figure 8(a)


White Cell Count
P<0.001
P<0.001
P<0.01
20
18
16
x10^9/L
14
12
10
8
6
4
N
IHD
AMI

Plasma Neutrophil Elastase

Figure 8(b)

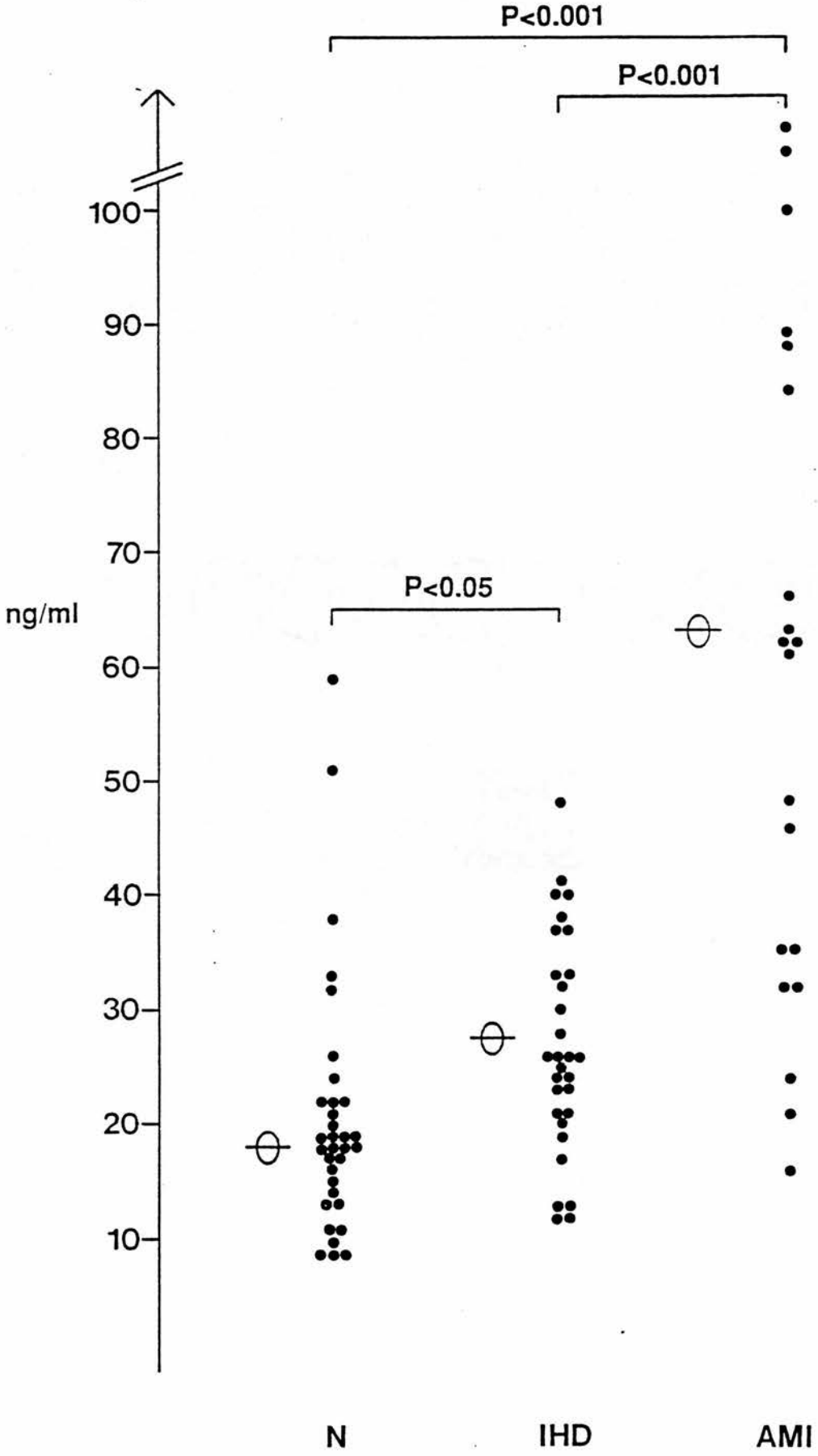
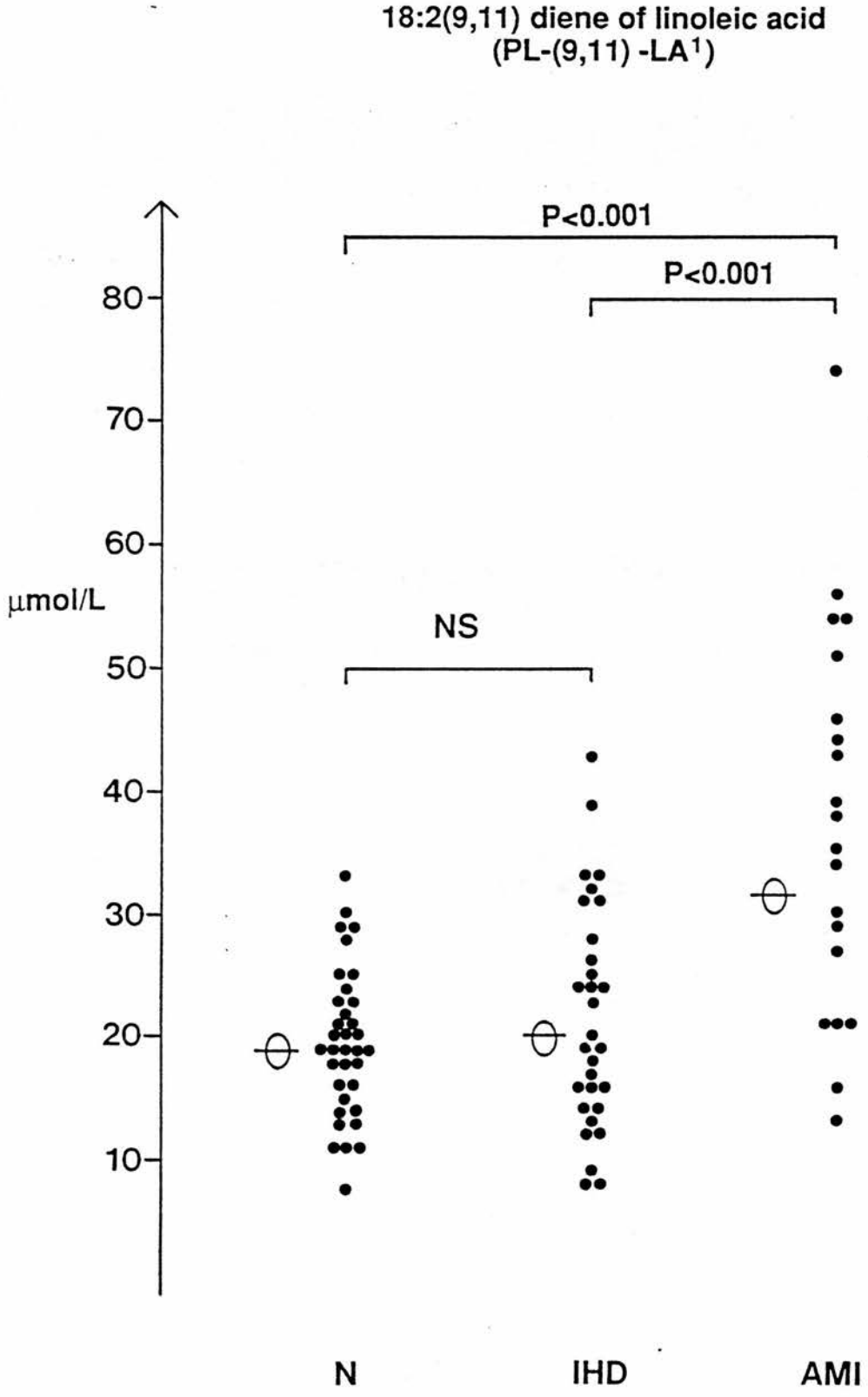


Figure 8(c)


18:2(9,11) diene of linoleic acid
(PL-(9,11) -LA[1])
P<0.001
P<0.001
NS
80
70
60
µmol/L
50
40
30
20
10
N
IHD
AMI

8.4 DISCUSSION

The results of this study confirm that patients with chronic ischaemic heart disease have an elevated leucocyte count. This agrees with work by Kostis, who reported that raised white cell counts correlated with the severity of coronary artery disease (based on coronary angiography) in a series of 573 patients (Kostis **et al**, 1984). An elevation of peripheral leucocyte count has also been shown to correlate with the risk of stroke, myocardial infarction and subsequent reinfarction (Friedman **et al**, 1974; Ernst **et al**, 1987; Lowe **et al**, 1985). The risk of myocardial infarction in particular is thought to increase by a factor of four in subjects with white cell counts greater than $9x10^9/l$ (Ernst **et al**, 1987).

Whilst smoking also increases the risk of myocardial infarction, only 50% to 65% of the increased risk in those with a high leucocyte count can be accounted for by smoking. In the patients with chronic stable heart disease only two admitted to smoking, although 10 had a previous history of tobacco use. Moreover as neither carboxyhaemaglobin nor cotinine were measured at the time of study it was difficult to gauge the contribution current smoking may have made to the leucocyte counts.

The elevation of the leucocyte count in patients with acute myocardial infarction occurred early after the event and fell over the ensuing period of the study. This was due to the more general response to stress with the mobilisation of the marginated leucocyte pool (Bierman **et al**, 1952).

Neutrophil elastase is released from the azurophilic granules of neutrophils following cell activation. The polyclonal antiserum against

purified human neutrophil elastase did not bind to any other neutrophil protein and did not detect the antigenically distinct elastases of platelets and pancreas. It did however measure neutrophil elastase equally well in the free form and when complexed to its inhibitors alpha-1-proteinase inhibitor and alpha-2-macroglobulin, the forms in which it circulates in plasma.

Elastase measured in whole blood correlates with the total neutrophil count and is primarily a measure of the intracellular stores of this protease. Since neutrophil elastase in plasma does not correlate with either leucocyte count or neutrophil count, then elevated levels of plasma neutrophil elastase reflect an increase in neutrophil activation in the form of degranulation (Greer **et al**, 1989).

The concentration of neutrophil elastase in normal plasma is usually low (Plow, 1982) as shown here, but is elevated in the plasma of both the patients with chronic stable ischaemic heart disease and acute myocardial infarction.

The higher than baseline levels for ischaemic heart disease may reflect a prevalent state of neutrophil activation. Neutrophil elastase, used here as a marker of activation, can cause substantial tissue damage as its substrates include not only elastin but also collagen, proteoglycans and other basement membrane components (Janoff, 1985). As discussed earlier in Chapter 1, when neutrophils adhere to the vascular endothelium they can create a "protected microenvironment" at the interface between the neutrophil and the endothelial cell so that the neutrophil proteases may attack and degrade the vascular tissue while remaining inaccessible to its plasma protease inhibitors.

Several studies correlating vascular disease with elevated circulating plasma levels of neutrophil elastase have been reported.

These include pregnancy-induced hypertension, (Greer **et al** 1989), peripheral vascular disease (Weissman **et al**, 1980) and diabetes mellitus (Collier **et al**, 1989).

Therefore this association of the neutrophil count and atheromatous vascular disease, in this case ischaemic heart disease is consistent with neutrophil activation contributing to the pathogenesis of vascular disease.

Evidence that the neutrophil plays a major role in the extension of myocardial damage has been provided by several animal studies but has proved more difficilt in man.

Romson demonstrated that dogs rendered neutropenic by administration of antisera to canine neutrophils, evolved infarcts that were 43% smaller than those dogs treated with non-immune sera. Since there were no haemodynamic differences caused by either treatment for the two canine groups, the reduction could be attributed to the induced neutropenia (Romson **et al**, 1983). The neutrophil's role as a mediator of tissue injury extension was further consolidated by Mullane **et al**, (1984), who showed a decrease in infarct size in dogs by reducing the circulating neutrophil count by 60%. More recent work investigating the neutrophil's involvement in myocyte injury by Simpson **et al**, (1988) demonstrated that blocking leucocyte cell adhesion-promoting glycoproteins (Mol;CD11b/CD18) on the neutrophil membrane with appropriate monoclonal antibodies (antiMol; antiCD11b), reduced the experimental infarct size without altering blood pressure, heart rate or coronary blood flow.

These observations provide additional evidence to support the important role of inflammatory cells in extending myocardial injury beyond that caused by ischaemia itself.

The response to tissue injury includes the activation of complement, the formation of chemoattractants and activators of neutrophils and local release of mediators capable of causing tissue injury. One of the complement components formed is C5a which has been reported as a potential mediator of the inflammatory response to myocardial ischaemia. (Williams **et al**, 1981). Included in its actions on neutrophils are aggregation, chemokineses, chemotaxis, release of oxygen free radicals and degranulation with release of enzymes (Crawford **et al**, 1988).

This study demonstrates that the release of neutrophil elastase is significantly raised in patients with acute myocardial infarction. The peak levels of this protease were detected in plasma at 24 hours post-infarction, at a time consistent with the presence of an inflammatory infiltrate in the myocardium.

All mammalian cells are subject to free radical reactions which occur continuously in vivo. Oxidative stress in cells and tissue occurs when there is increased oxygen tension, and as a result of activation of enzyme systems may lead to the formation of O_2^- (Bellavite **et al**, 1988).

As discussed in Chapter 1, activated neutrophils are capable of generating reactive oxygen species through reduction of molecular oxygen by NADPH oxidase at the plasma membrane.

However neutrophils do not represent the only possible source of these highly reactive moieties. Recent studies (Chambers **et al**, 1985) suggest that xanthine oxidase may be an important source of free radicals within ischaemic tissue. During ischaemia, cytosolic xanthine dehydrogenase is converted to xanthine oxidase by limited proteolysis. This, rather than producing NADH as its reaction product, generates the superoxide radical via univalent reduction of oxygen. However, although

this system is known to exist in the vascular endothelium, its role in myocyte damage is still questioned as its presence has yet to be confirmed in myocardial specimens (Werns **et al**, 1987; Watts **et al**, 1965).

Therefore in man the most likely sources of free radical production in the myocardium are the electron transport chains in the myocyte mitochondria or from activated neutrophils via membrane linked NADPH oxidase (McCord, 1987).

PL-9,11-LA', the non-peroxide isomer of linoleic acid was used here as an in vivo marker of free radical activity (Iversen **et al**, 1985). The plasma levels of PL-9,11-LA' in patients with myocardial infarction were high initially and fell over the remaining 48 hours, suggesting early free radical activity.

Sources of PL-9,11-LA' are present in the diet and also have been shown to be manufactured by bacterial flora in the gut. Although feasible, it is unlikely that the diet or bacterial flora would contribute to the change in the PL-9,11-LA' observed in this relatively short 48 hour period following infarction.

This study demonstrates that increased lipid oxidation occurs in the early phase of myocardial infarction and is consistent with free radical activity in man. Neutrophil activation also occurs and may be implicated in potentiating myocardial injury after myocardial infarction.

The results of this study confirm that neutrophil activation and increased free radical activity occurs in man in the 48 hour interval after acute myocardial infarction.

CHAPTER 9

EFFECTS OF THROMBOLYSIS ON PNE, PL-9,11-LA' AND ^{111}INDIUM IMAGING IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION

9.1 INTRODUCTION

As early as 1912, in describing the syndrome of acute myocardial infarction, Herrick suggested that "hope for the damaged myocardium lies in the direction of securing a supply of blood so as to restore as far as possible its functional integrity" (Herrick **et al**, 1912). In the 1930s several groups (Tennant **et al**, 1935; Blumgart **et al**, 1940) studied coronary occlusion and the effect of its duration in canine studies. Results highlighted the benefits of early restoration of blood flow to the ischaemic tissue in resolving abnormalities in myocardial contraction and in some cases infarction was avoided. Further studies in the 1970's by several groups (Smith **et al**, 1974; Theroux **et al**, 1976) showed, in animal models of infarction, that early reperfusion limited the damage to the heart and also greatly improved left ventricular function.

These observations led to the evaluation of strategies in man to re-establish blood flow early in the evolvement of infarction. Originally these were aimed at reducing myocardial oxygen demand (Constantini **et al**, 1975) but these treatments did not demonstrate benefit and as a result failed to gain support. Similarly, failure to show convincing benefit with heparin and inconclusive results with streptokinase led to the examination of percutaneous transluminal angioplasty, surgical revascularisation and thrombolysis as vehicles for reperfusion. Both angioplasty and revascularisation are technically difficult and neither has been shown to be of major value.

In contrast numerous clinical trials have shown the benefit of the use of thrombolysis. Ischaemic myocardium, which may otherwise become irreversibly damaged, may be, at least partly salvaged by timely reintroduction of the hearts blood supply by dissolution of thrombus as a result of intracoronary or intravenous administration of a variety of thrombolytic agents.

The use of thrombolytic agents for this purpose is now common practice (GISSI, 1986; TIMI, 1985) and although experimentally it is difficult to show what is happening to myocardium, results from clinical trials show that patients treated with thrombolytic therapy have lower immediate and longer term mortality rates.

Despite this, there is considerable interest in the concept of reperfusion injury (Werns **et al**, 1986). Early fears surrounding this treatment were based on studies in animals. Following administration of the thrombolytic agent, reperfusion was found to be associated with development of ventricular arrhymias, myocardial 'stunning', microvascular damage, cell necrosis and haemorrhage (Anon. 1989). Postulated mechanisms of reperfusion injury include; the no-reflow phenomena, increased free radical production and neutrophil activation with concomitant release of lysosomal enzymes (Braunwald **et al**, 1985).

The value of thrombolysis to the clinical course of the patient with myocardial infarction heavily outweighs these potentially harmful side-effects. Subtle changes in the reperfused myocardium are difficult to characterise in vivo, however if they exist, reduction of any deleterious effects would be desirable.

Successful imaging of the migration of neutrophils into infarcted myocardium was described in Chapter 7 and evidence of increased neutrophil activation and free radical activity accompanying the acute

inflammatory response to myocardial infarction in 20 patients treated conventionally was described and characterised in Chapter 8.

The aim of this part of the study was to assess the effect of intravenous administration of thrombolytic agents on the inflammatory response by measuring plasma neutrophil elastase, the non-peroxide isomer of linoleic acid (PL-9,11-LA') and imaging the cellular response in a group of patients with myocardial infarction who received thrombolysis and comparing them to a further group who were treated conventionally. In addition these patients were compared the previously defined (Chapter 8) normal controls and patients with stable ischaemic heart disease.

9.2 MATERIALS AND METHODS

9.2.1 SUBJECTS STUDIED

CONTROL GROUPS

The control group of healthy subjects (N; n=35) and the group of patients with a documented history of stable ischaemic heart disease (IHD; n=30), were described in Chapter 8.

ACUTE MYOCARDIAL INFARCTION

Clinical trials have conclusively shown benefit of thrombolytic therapy to patient health. It was therefore considered unethical to deny patients this treatment where indicated and consequently randomisation of patient therapy was not considered appropriate in this study.

A group of 32 patients with acute myocardial infarction were studied. Diagnosis was made on evidence of prolonged chest pain (duration longer than 30 minutes), characteristic ECG changes and an increase in creatine kinase to at least twice the normal value.

This patient group was further divided according to medical treatment received while in coronary care unit (CCU). A sub-group of patients received thrombolytic therapy (n=17), either by administration of 1 200 000 units of streptokinase (Kabivitrum, Middlesex) or 30 units anisoylated plasminogen streptokinase complex (APSAC, Beechams Pharmaceuticals, Epsom, UK). The remaining 15 patients were judged unsuitable to receive thrombolysis and so were treated conventionally. Reasons for excluding thrombolytic therapy included late admission (after 4 hour of chest pain), patient history of peptic ulcer, bleeding episodes or recent cerebrovascular event.

Patients in this group had venous blood taken for estimation of plasma neutrophil elastase (PNE), the non-peroxide isomer of linoleic

acid in the phospholipid fraction of plasma (PL-9,11-LA'), peripheral white cell count (WBC) and creatine kinase (CK). The initial samples were taken within 6-8 hours of admission and every 6-8 hours thereafter in the first 48 hours after the major onset of chest pain. The time of sampling was relative to the best estimate of the time of the major onset of chest pain. The protocol of the blood sampling regime is shown in Figure 9(a).

In addition to measuring these markers of neutrophil activation and free radical activity, the acute inflammatory response to myocardial infarction was imaged in 21 of the 32 patients. Venous blood (60ml) was taken from 11 of the patients receiving thrombolytic therapy and 10 of those being treated conventionally. The methods used for isolation of neutrophils from whole blood and labelling with ^{111}Indium oxine are described in Chapter 2.

9.2.2 Plasma Neutrophil Elastase (PNE)

Plasma neutrophil elastase was measured in citrated plasma using a standard specific radioimmunoassay which employed rabbit polyclonal antiserum, (Greer **et al**, 1989) and is described in Chapter 2. The results are expressed as ng/ml and the intra-assay coefficient was less than 5%.

9.2.3 PL-9,11-LA' and PL-9,12-LA

The molar concentrations of PL-9,11-LA' and PL-9,12-LA (linoleic acid) in heparinised plasma were measured by high performance liquid chromatography (HPLC) in plasma after enzymatic hydrolysis with phospholipase A_2 and solid phase sample preparation by the method of Iverson **et al**, (1985), described in Chapter 2. The intra-assay

coefficient was less than 3.5%. Results are expressed as umol/l.

9.2.4 White Cell Count (WBC) and Creatine Kinase (CK)

Peripheral white cell counts were performed on a sequestrene sample using a Sysmex E5000 (Toa Electronics Ltd., Kobe, Japan). Creatine kinase (CK) in heparinised plasma was measured by the hospital clinical chemistry department.

9.2.5 IMAGING

9.2.6 Neutrophil Uptake

Due to the limited availablity of the radioisotope and the clinical problems associated with imaging patients in the acute phase of infarction, 21 of the 32 patients were included in this part of the study. Eleven patients who had received thrombolytic therapy and 10 treated conventionally had blood taken for neutrophil isolation and labelling. (Methods described in Chapter 2).

It was shown in Chapter 7 that using ^{111}Indium labelled autologous neutrophils, the inflammatory response that accompanies acute myocardial infarction could be reliably imaged provided the labelled cells were injected within 18 hours of the onset of chest pain.

All 21 patients were reinjected with ^{111}Indium labelled neutrophils (Median Dose 25MBq; Range (18-51 MBq)) within 18 hours (9 hours (5-17 hours))of initial chest pain.

Single photon emission tomography (SPET) was performed on each patient 24 hours after injection of the labelled neutrophils, using a IGE 400AT Maxicamera linked to a Seimens Microdelta Computer. (See Figure 9(b). A series of 64 images were acquired during 360° rotation around the patient. These were constructed by back projection using a

Butterworth filter to create saggital, transverse and coronal views. A study was considered positive when ^{111}Indium uptake in the area of the heart was seen in all three views.

9.2.7 Infarct Sizing

The following day SPET imaging was used to image the size of the infarct in the same patients. Each received an intravenous dose of 500MBq of technetium-99m pyrophosphate (^{99m}Tc-PYP) and imaged 2 hours later using the same procedure as described for neutrophil uptake (described above).

9.2.8 Estimation of the Volume of Uptake of Neutrophil Infiltrate compared to Infarct Volume

For each patient the uptake of ^{99m}Tc-PYP and ^{111}Indium labelled neutrophils in all the transverse slices with myocardial uptake were analysed using a semi-automated program which counted the number of volume cell elements (VOXELS) with values 65% of the peak myocardial uptake (Jansen **et al**, 1985). Using this method the volume of the heart showing neutrophil uptake and the volume of infarcted myocardium, as judged by the pyrophosphate images, may be compared.

9.2.9 Radionuclide Ventriculography

Each of the infarct patients had radionuclide ventriculography performed to assess left ventricular function. Ejection fraction was assessed at equilibrium using multiple gated acquisition method (MUGA).

^{99m}Technetium-human serum albumin (740MBq) was prepared from a freeze-dried kit (TCK-2, CIS (UK) Ltd) and injected intravenously into

the antecubital fossa. At equilibrium the gamma camera head was positioned in the left anterior oblique (LAO) position to isolate the left ventricle. A series of 24 images were acquired (approximately $5x10^6$ counts), triggered by the R wave of the ECG. A region of interest was drawn around the left ventricle and the ejection fraction (EF) calculated from:

EF= diastolic counts-systolic counts/diastolic counts x 100%

9.2.10 Statistical Analysis

The data were found not to be normally distributed, as tested by the Kolmogorov-Smirnov test. Results are expressed as the median and range. The data were compared using non-parametric analysis using the Wilcoxon Rank Sum Test for two independent samples (Mann-Whitney). Values of p 0.05 were taken as significant. The statistics were performed by computer using a statistical package for social sciences (SPSS, Chicago).

9.3 RESULTS

9.3.1 WHITE CELL COUNT

The white blood cell count was significantly lower in the normal controls ($5.8x10^9$/l, 3.4-9.3) than in the patients with stable ischaemic heart disease ($6.7x10^9$/l, 5.2-12.6, $p<0.004$) and this was also true for the initial white cell count for the patients with myocardial infarction ($16.4x10^9$/l, 7.9-33.0, $p<0.0001$).

The white blood cell count was significantly lower in the group with stable ischaemic heart disease compared to the patients with acute myocardial infarction ($p<0.0001$).

9.3.2 PLASMA NEUTROPHIL ELASTASE

The concentration of neutrophil elastase in plasma for normals (18.6ng/ml, 9.2-51.0) was significantly lower than that for the patients with stable ischaemic heart disease (25.8ng/ml, 12.2-49.5, $p<0.002$).

In the patients with acute myocardial infarction, plasma neutrophil elastase was significantly higher than both the ischaemics and the normal controls (see Figure 9(c)) throughout the 48 hour period after infarction.

9.3.3 PL-9,11-LA' and PL-9,11-LA'/PL-9,12-LA

There was no significant difference in PL-9,11-LA' or the molar ratio PL-9,11-LA'/PL-9,12-LA between the normal controls (19.3umol/l, 7.5-32.9; 4.7, 1.9-9.1) and the patients with ischaemic heart disease (19.8umol/l, 7.9-43.2; 5.4, 1.7-12.1).

The values of PL-9,11-LA' for the patients with myocardial infarction at all times over the 48 hour period were significantly higher than both

the controls and the ischaemic group. This was also true for the molar ratio of PL-9,11-LA'/PL-9,12-LA compared to the control group, however the ratio was significantly higher than the ischaemics only during the first 24 hours following infarction (Figures 9(d) and 9(e)).

9.3.4 **Comparison Within the Patient Group with Myocardial Infarction Thrombolysis and Conventional Treatment**

9.3.5 White Cell Count (WBC)

There was no difference in white cell count between the non-thrombolytic group (13.2×10^9/l, 9.6-23.0) and the patients who received thrombolytic therapy (16.8×10^9/l, 7.9-33.7).

9.3.6 Plasma Neutrophil Elastase (PNE)

The pattern of change in levels of elastase in plasma was different in the two treatment groups (Figure 9(f)). Plasma neutrophil elastase for the patients who received thrombolytic therapy peaked at 8 hour (48.2ng/ml, 25-250) and was significantly higher than the non thrombolytics (32.6ng/ml, 15.6-101, $p<0.025$) at this time. Over the remaining period the elastase values fell slowly.

Those patients treated conventionally tended to have low early values and the peak (49.8ng/ml, 21.4-196) occurred later, at around 40 hours post infarct and was significantly higher than those treated with thrombolysis (34.2ng/ml, 15.8-83, $p<0.037$).

9.3.7 PL-9,11-LA' and PL-9,11-LA'/PL-9,12-LA

There was no significant difference in PL-9,11-LA' or the molar ratio PL-9,11-LA'/PL-9,12-LA in those treated conventionally after myocardial infarction and those given thrombolysis. For both groups the values were maximal at 16 hours and gradually fell to normal levels. (See Fig 9(e) and 9(h))

9.3.8 Plasma Creatine Kinase (CK)

There was no significant difference in peak creatine kinase between patient treated conventionally (1635U/l, 522-6255) and those who were treated with thrombolysis (2059U/l, 533-6955). The plasma activity of creatine kinase reached a peak earlier in those treated with thrombolysis (16 hours) than in those treated conventionally (24 hours).

9.3.9 Left Ventricular Ejection Fraction (LVEF)

There was no significant difference in the LVEF measured by radionuclide ventriculography 10 days after infarction, though those patients who were treated with thrombolytic agents (40%, 24-68) had slightly higher values than those who were treated conventionally (32%, 15-71).

9.3.10 Imaging

There was no significant difference in the peak creatine kinase, LVEF and time to reinjection of autologous radiolabelled neutrophils for patients treated conventionally and those treated with thrombolytic therapy (see Table 9(ii)).

Uptake of ^{99m}Tc pyrophosphate was similar for the two treatment groups suggesting little difference in the size of infarcts experienced by these patients. This is further supported by the similarity of the peak

creatine kinase for both. The uptake of ^{111}Indium labelled neutrophils was less in the patients treated with thrombolysis.

The ratio of $^{111}In/^{99m}Tc$ serves as an estimate of the inflammatory response for a given infarct, and this was significantly less in patients treated with thrombolysis (0.41, 0-0.96) than in those treated conventionally (0.79, 0.06-2.14, $p<0.05$).

Figure 9(i) shows an example of the reduced uptake of ^{111}Indium labelled neutrophils in a patient treated with streptokinase compared with that seen in a patient who received no thrombolysis.

9.4 **Influencing Factors**

There was no correlation between the leucocyte count and the plasma concentration of neutrophil elastase in any of the groups studied. There was a weak correlation between the leucocyte count and PL-9,11-LA' in the non-thrombolytic group ($r=0.63$;$p<0.02$) and in the patients with ischaemic heart disease ($r=0.45$; $p<0.02$) but this did not hold for the corrected molar ratio and no correlations were found in the normal volunteers and patients treated with thrombolysis.

9.4.1 Acute Myocardial Infarction

There was no correlation of creatine kinase with PL-9,11-LA' or the molar ratio of PL-9,11-LA'/PL-9,12-LA measured in the same sample. Creatine kinase did not correlate with the increase in plasma neutrophil elastase seen in either group of patients whether treated with thrombolytic therapy or not.

LVEF did not correlate with PNE, PL-9,11-LA' or the molar ratio of PL-9,11-LA'/PL-9,12-LA.

TABLE 9(i)

Details of healthy volunteers (controls), patients with chronic ischaemic heart disease (IHD), and patients with myocardial infarction (AMI).

			Myocardial Infarction	
	Controls	IHD	No Thrombolysis	Thrombolysis
	(n=35)	(n=30)	(n=15)	(n=17)
M/F	30:5	24:6	10:5	13:4
Age	34	59	58	56
	(22-63)	(37-76)	(38-74)	(30-69)
WBC($x10^9$/l)	5.8	6.7	13.2	16.8
	(3.4-9.3)	(5.2-12.6)	(9.6-23.0)	(7.9-33.7)
LVEF (%)		41	32	40
		(18-63)	(15-71)	(24-68)
Peak CK(U/l)			1635	2059
			(522-6255)	(533-6955)
Heparin (sub)			15	-
Heparin (i.v.)				17
Streptokinase/anistreplase				9/8
Lignocaine			3	5
Hydrocortisone				9
Beta-blockers		24	2	5
Diuretics		4	6	5
Deaths			2	1

TABLE 9(ii)

Details of patients treated with and without thrombolysis who were imaged with ^{111}Indium-labelled neutrophils and ^{99m}Technetium-pyrophosphate. Data are expressed as mean(range).

	No thrombolysis (n=10)	Thrombolysis (n=11)	p value
CK (max) (U/l)	2508 (522-6255)	2500 (533-6955)	NS
LVEF (%)	36 (15-51)	27 (27-68)	NS
Time to injection (h)	8 (6.5-10)	11 (5-18)	NS
^{111}Indium (voxels)	114 (19-276)	81 (0-160)	NS
^{99m}Technetium (voxels)	201 (77-405)	217 (111-323)	NS
^{111}In/^{99m}Tc	0.79 (0.06-2.14)	0.41 (0-0.96)	0.05

CK creatine kinase, LVEF left ventricular ejection fraction

PROTOCOL

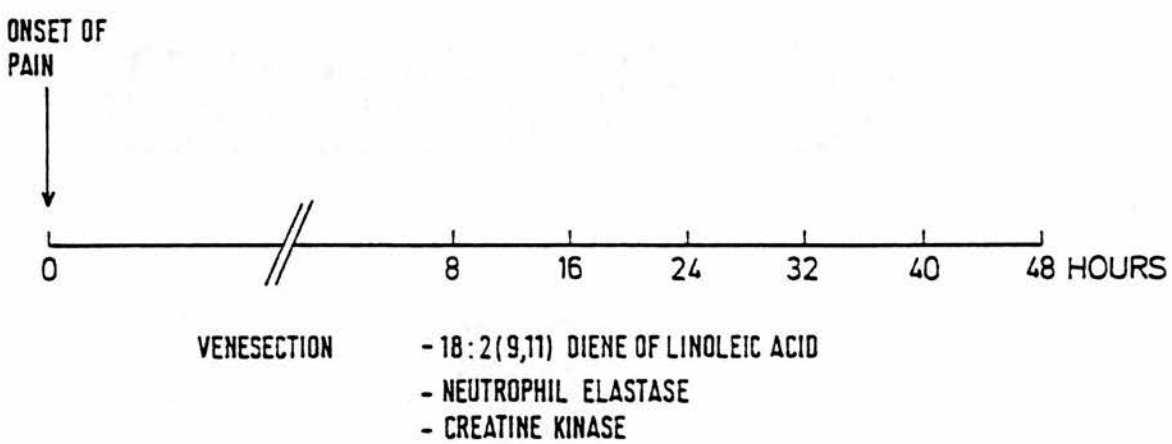
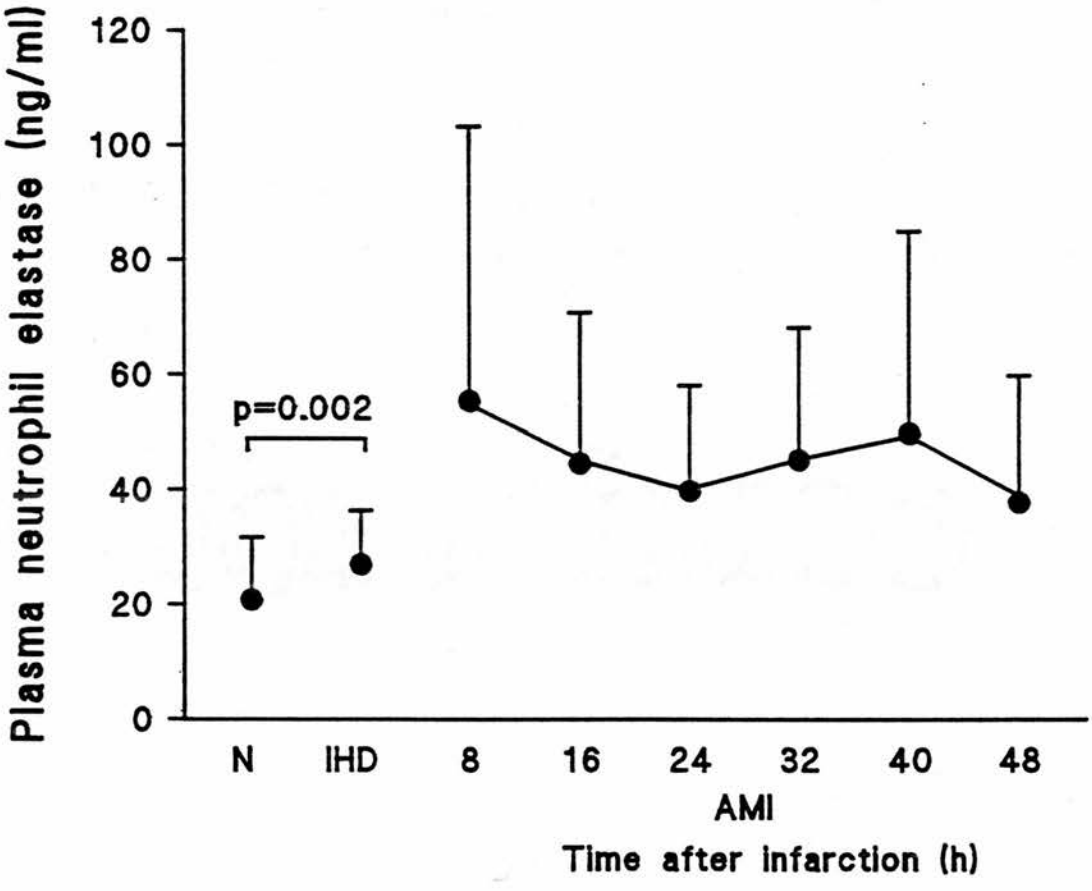


Figure 9(a)

Figure 9(b)

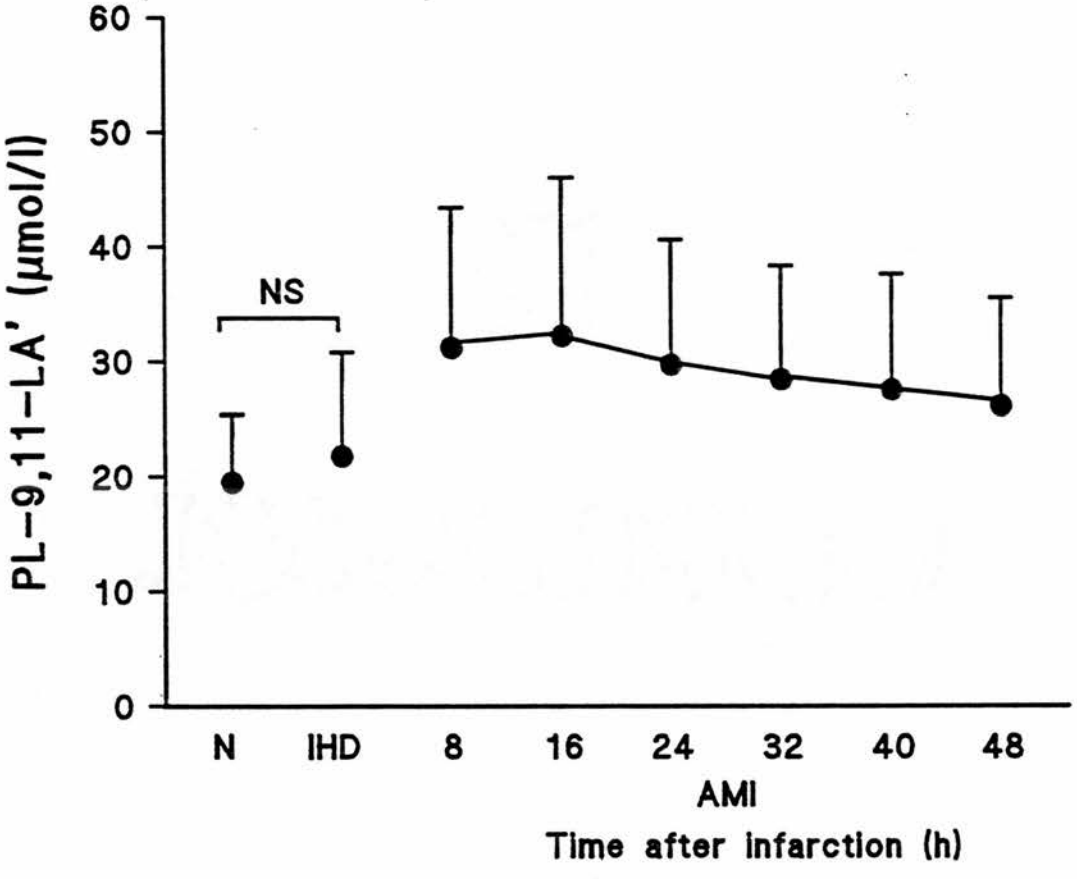
IGE 400AT Maxicamera linked to a Seimans Microdelta Computer showing a volunteer in position for SPET imaging.

Figure 9(c.)



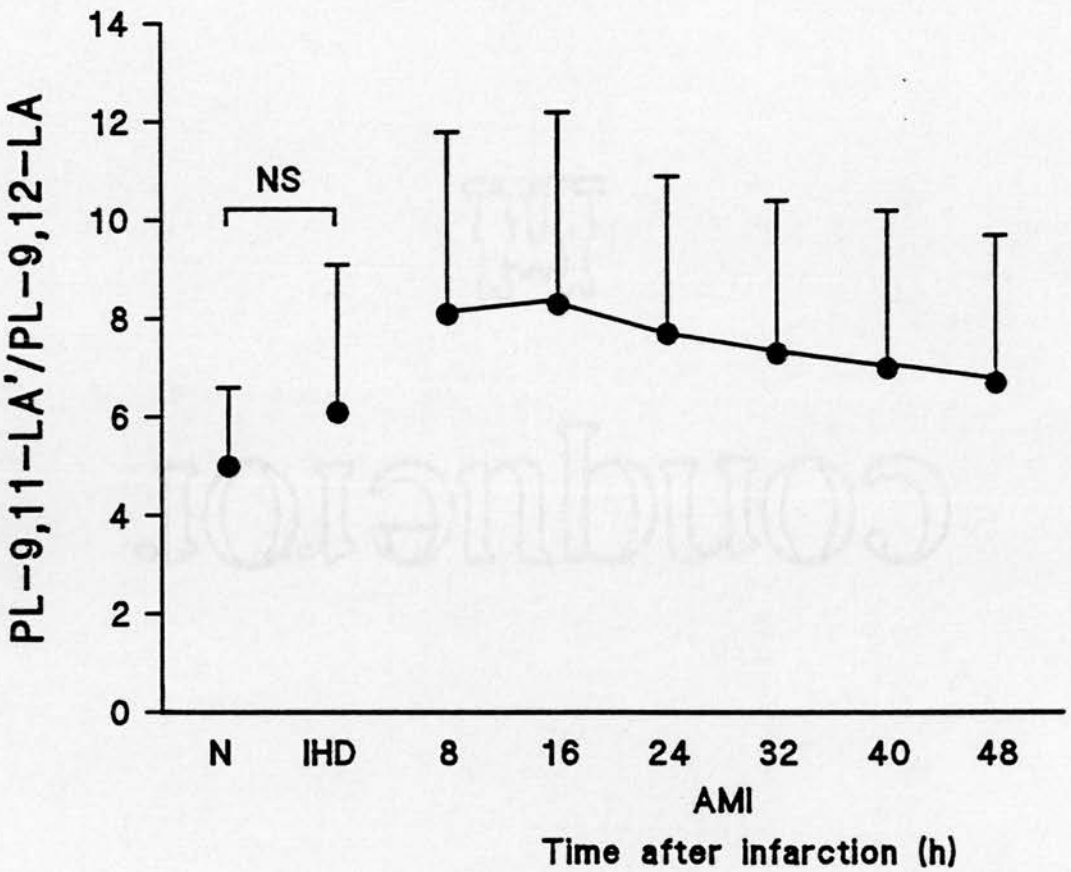
Plasma neutrophil elastase for normals (N), patients with ischaemic heart disease (IHD) and acute myocardial infarction (AMI)

Figure 9(d)



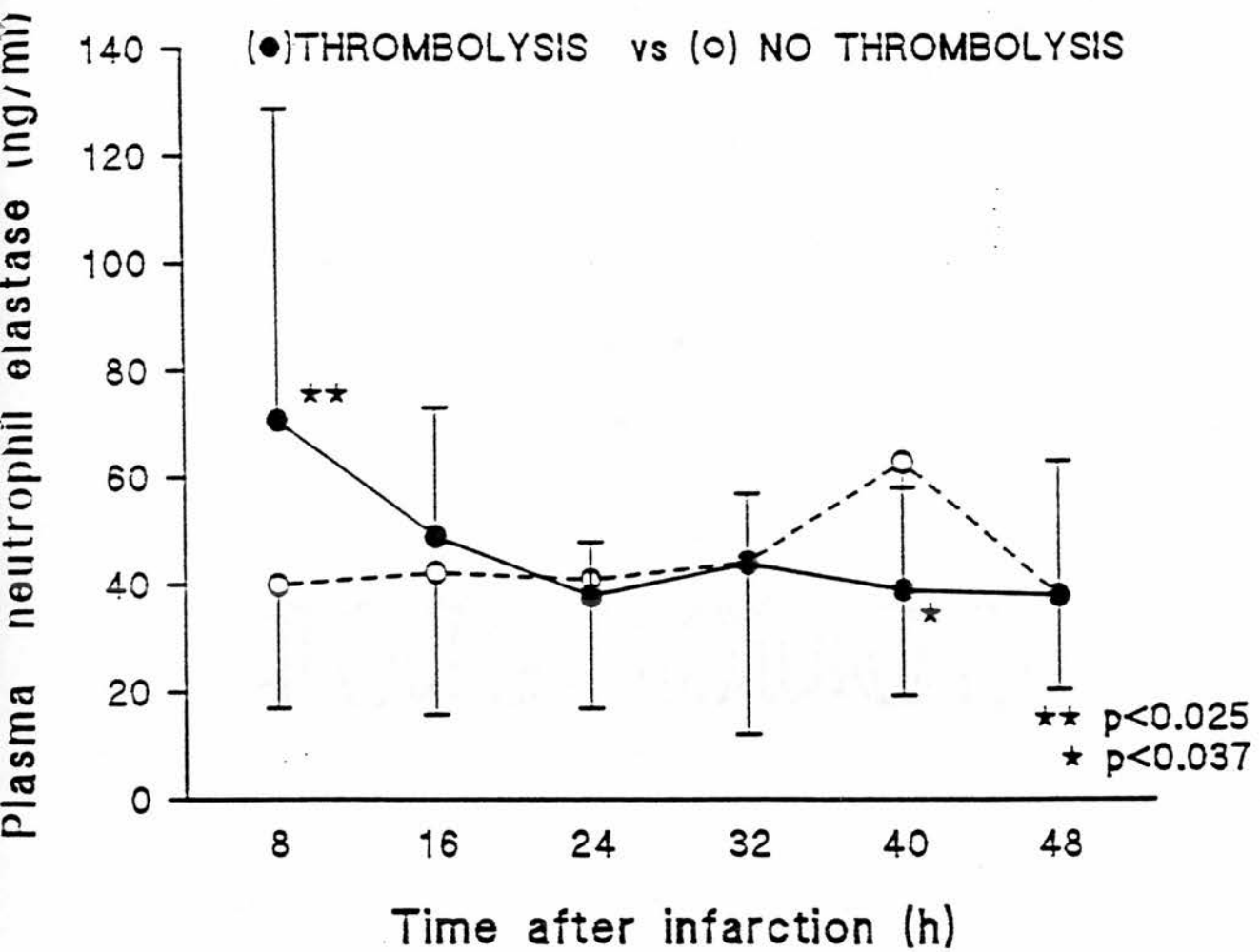
PL-9,11-LA' for normals (N), patients with ischaemic heart disease (IHD) and acute myocardial infarction (AMI)

Figure 9(e)



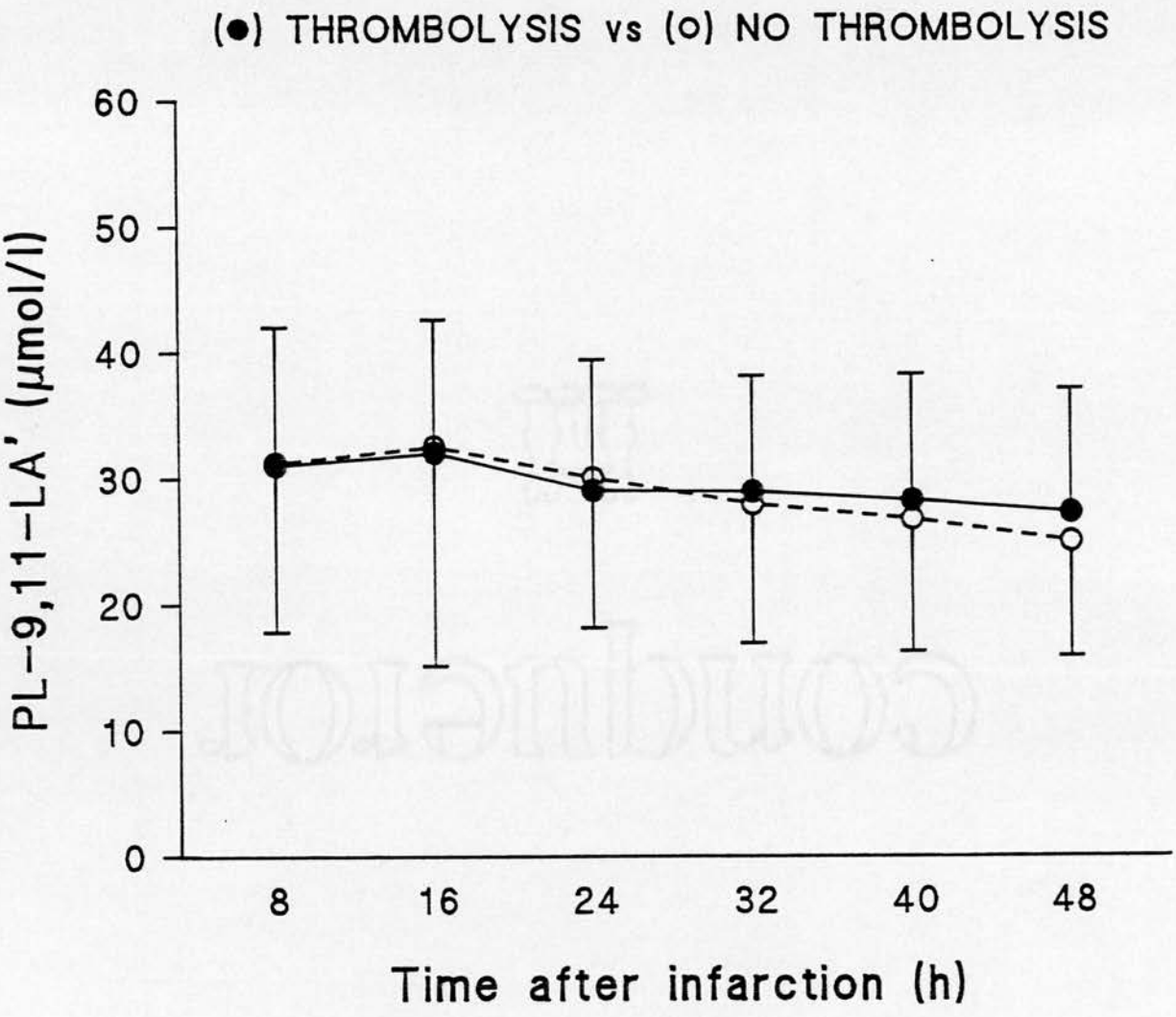
PL-9,11-LA'/PI-9,12-LA for normals (N), patients with ischaemic heart disease (IHD) and acute myocardial infarction (AMI)

Figure 9(f)



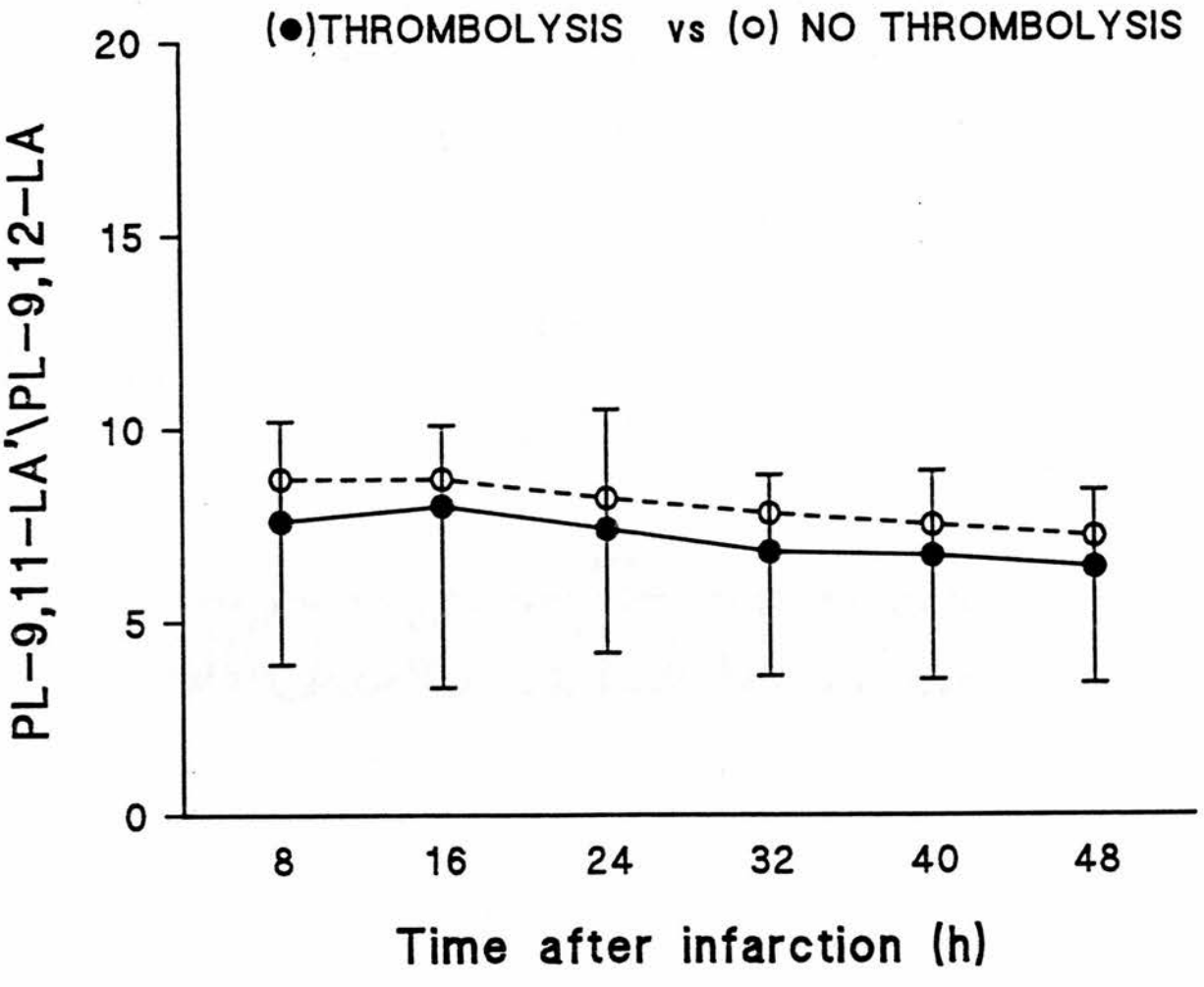
Plasma neutrophil elastase for patients with myocardial infarction treated conventionally (○) or with thrombolytic therapy (●).

Figure 9(g)



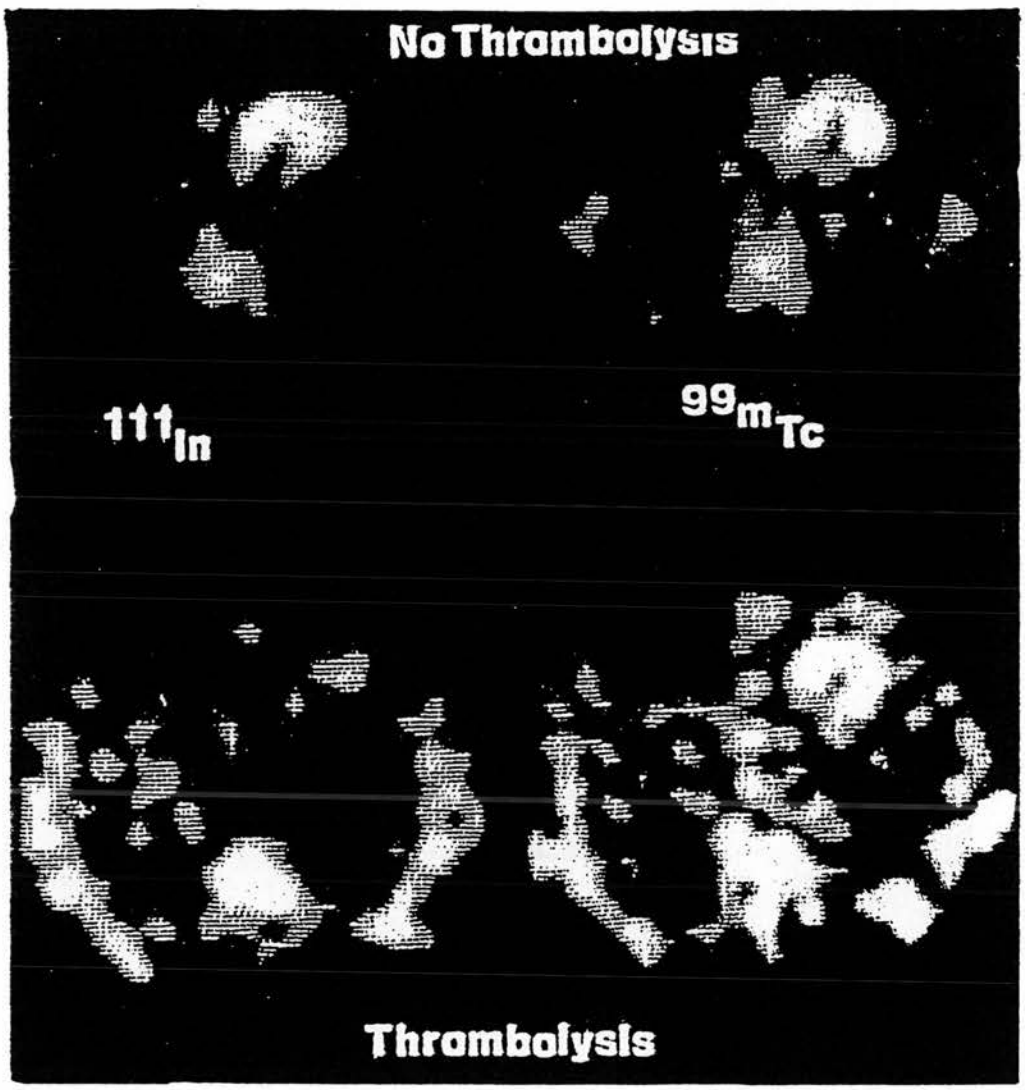
PL-9,11-LA' for patients with myocardial infarction treated conventionally (o) or with thrombolytic therapy (●).

Figure 9(h)



PL-9,11-LA'\PL-9,12-LA for patients with myocardial infarction treated conventionally (o) or with thrombolytic therapy (●).

Figure 9(i)



SPET images comparing the uptake of ^{111}In labelled neutrophils with the size of infarction as assessed by ^{99m}Tc-PYP. The upper pair of images show easily detected uptake of ^{111}In and ^{99m}Tc-PYP in a patient with anterior AMI who was not given thrombolytic treatment. The lower pair of images show markedly reduced uptake of ^{111}In labelled neutrophils compared with ^{99m}Tc-PYP in a patient with anterior AMI treated with anistreplase.

9.5 **DISCUSSION**

This study confirms the findings of Chapter 8 that statistically significantly raised levels of neutrophil elastase and PL-9,11-LA' indicating increased neutrophil activation and free radical production respectively, exist in the plasma of patients following acute myocardial infarction when compared to normal subjects and a further control group of patients with stable ischaemic heart disease. Moreover, the pattern of production of these species differs in patients with acute myocardial infarction according to whether or not thrombolytic therapy has been administered. More importantly, reperfusion did not appear to be associated with an exacerbated production of these potentially harmful species.

The clinical benefits of thrombolysis after myocardial infarction are now well documented (GISSI-2, 1990). The dramatic decrease in patient mortality, coupled with improvement in left ventricular function has more than proved the case for its use where clinically indicated. Naturally, as with all novel therapies, and particularly one which has received such close scrutiny by the medical profession, there has been considerable debate on the balance between the beneficial and possibly detrimental effects of such treatment. Complications of reperfusion were first indicated in studies on animals and was found to be associated with development of ventricular arrhymias, haemorrhage and less obvious abnormalities such as myocardial stunning, microvascular damage and cell necrosis. The problems of arrhymia and bleeding have been monitored in a number of studies (ISIS-2, 1988; AIMS, 1990), however hastening of myocyte necrosis with extension of myocardial injury and several other phenomena are much more difficult to quantify in man. 'Reperfusion Injury' due to early reintroduction of oxygen into the ischaemic tissues

has been supposed to occur through several possible mechanisms. Braunwald in addressing this problem postulated the involvement of several factors which include the 'no-reflow' phenomenon, the calcium and oxygen paradox and the involvement of leucocytes (Braunwald **et al**, 1985).

Markers of free radical production and neutrophil activation were measured in two groups of patients with myocardial infarction and the effects of thrombolysis on these parameters was also studied.

Plasma neutrophil elastase was maximal in the period between 32 and 40 hours after myocardial infarction in patients treated conventionally. This coincides with the presence of the inflammatory infiltrate which is known to be present histologically (Sommers **et al**, 1964) at this time. In contrast, in patients treated with thrombolytic agents (either streptokinase or Anistreptlase) there was an early and significant increase in plasma neutrophil elastase within the first few hours of treatment. This agrees with previous reports (Gutteridge **et al**, 1988) of early neutrophil activation. This early peak of plasma neutrophil elastase may reflect clot lysis at the time of thrombolysis or intracoronary activation of neutrophils, as few will be present in the myocardial tissue at this early stage (Mallory **et al**, 1939). It is of note that the elevation of neutrophil elastase seen in the plasma of the conventionally treated patients in the period which appears to concur with the presence of the inflammatory infiltrate, is absent in this same period after myocardial infarction in patient group who received thrombolysis.

Differences in drug regime, other than that due to the administration of thrombolytic agents may be considered as a possible source for the disparity in results. The administration of lignocaine,

an anti-arrhythmic, may be considered as an influencing factor in view of the known in vitro (Goldstein **et al**, 1977) and potential in vivo effects (de Lorgeril **et al**, 1988). However, similar numbers of patients in each group received lignocaine. Also in the thrombolytic group, patients who received hydrocortisone prior to receiving streptokinase, did not have reduced uptake of ^{111}Indium or lower elastase concentrations than the patients treated with Anistreplase, who did not receive hydrocortisone. This suggests that the results seen in the thrombolysis group were not influenced by the potentially suppressive effect of hydrocortisone on the inflammatory response and neutrophil function (Hart **et al**, 1984).

Therefore the absence of the later peak of neutrophil elastase might suggest that administration of thrombolysis may in fact suppress the cellular inflammatory response.

This hypothesis is supported by the results of imaging. Of the 21 patients who were imaged with autologous ^{111}Indium labelled neutrophils only 2 of these showed no uptake of activity in the area of the heart. Although this may be due to a failure of cell labelling or imaging procedures, this is unlikely, since all subjects received the labelled cell preparation within the prescribed time following onset of chest pain thus ensuring a positive result ie. within 18 hours of chest pain. The two patients who had negative scans received thrombolysis and since all of this group had significantly reduced uptake of neutrophils in relation to infarct size, (assessed by ^{99m}Tc-PYP) it is more likely that the response was reduced to such an extent that indium activity was indetectable.

Increased free radical activity, measured indirectly as the isomerised diene conjugate of a membrane lipid in the phospholipid

fraction of plasma of both infarct groups, was confirmed. The values of PL-9,11-LA' and the molar ratio of its substrate linoleic acid, were initially high and then fell over the 48 hours following myocardial infarction suggesting early maximal free radical activity. The conventionally treated and the thrombolytic groups followed a similar trend and no statistical differences in the levels could be detected over the period of the study. This similar pattern of free radical generation for the two groups, with the thrombolytic group showing slightly lower values, suggests that in this study, reperfusion, induced by thrombolysis was not associated with a delayed or further increase in free radical production. Neither the concentration of PL-9,11-LA' nor the molar ratio correlated with creatine kinase measured in the same sample and it therefore seems unlikely that the raised values were merely a marker of cell damage (Halliwell **et al**, 1984).

The results from this study support the concept of a reduction in the acute inflammatory response to acute myocardial infarction by treatment with thrombolytic therapy.

The conventionally treated patient group showed an early and continued elevation in free radical activity. This was coupled with a sharp increase in neutrophil activation, and was coincident with the presence of the infiltrate associated with the inflammatory response to ischaemic tissue as evidenced by the radiostopic studies. A rise in free radical activity similar temporally but slightly reduced was seen in the thrombolytic group. If the early peak of neutrophil elastase detected in this group represents clot lysis or intracoronary activation at a time when the neutrophil numbers present in the tissue will be neglible, then the absence of the later peak seen in the previous group would suggest a reduction in the infiltrate, a point also demonstrated in this work by

the attenuation of ^{111}Indium-labelled neutrophils into the area of infarct.

If late myocyte injury does occur after myocardial infarction it may be mediated by the release of proteolytic enzymes from the large number of neutrophils present at the height of the inflammatory response rather than free radicals. The presence of these species was confirmed by the continued increase in lipid oxidation. The ability of free radical products to inactivate one of the main regulatory inhibitors of neutrophil elastase ie. alpha-1-antiproteinase by oxidation of its reactive centre is well documented, (Weiss, 1989) and may further potentiate tissue injury by the neutrophil.

In animals, administration of free radical scavengers such as superoxide dismutase (SOD) has been successful in reducing the extent of myocardial damage (Schlafer **et al**, 1982) and there is evidence that this agent may work in part by reducing neutrophil migration (McCord **et al**, 1982). It may be possible, therefore that part of the beneficial effect of the administration of thrombolytic therapy post-infarction may lie in the partial inhibition of the inflammatory response to myocardial injury, perhaps by reduction in the extent or duration of the production of neutrophil chemoattractants, such as C5a or LTB_4 (Hartmann **et al**, 1977).

This study has shown that after myocardial infarction neutrophil activation and free radical production occurs. Reperfusion, following successful thrombolysis did not appear to associated with an amplification of the inflammatory response of prolonged free radical production. Rather, these were somewhat diminished and it may be that some of the benefits of thrombolysis may be due to down regulation of the acute inflammatory response.

CHAPTER 10

CONCLUSIONS

Acute myocardial infarction in man is one of the commonest causes of mortality in the western world.

Evidence from experimental models of infarction suggest that the neutrophil may exert additional harmful effects on the surrounding myocardial tissue (Romson **et al**, 1983; Jolly **et al**, 1986).

The aim of this thesis was to determine if the neutrophil played a significant role in acute myocardial infarction in man. To study neutrophil activity, a method to isolate and radiolabel autologous neutrophils was developed. This, in conjunction with established markers of neutrophil activation and free radical activity, was used to determine neutrophil involvement in acute myocardial infarction. Particular focus was then made on patients who received coronary thrombolysis as "Reperfusion Injury" has been frequently been demonstrated in animal studies (Engler **et al**, 1983).

This final chapter will discuss to what extent these aims have been fulfilled and how research in this field has developed during the last few years.

Studies in animal studies, have shown that the inflammatory response may be monitored in vivo by administration of autologous radiolabelled leucocytes with subsequent imaging (Thakur **et al**, 1979; Weiss **et al**, 1977). To assess whether the neutrophil played a significant role in myocardial infarction in man, methods for neutrophil isolation and labelling were developed.

Although methods for neutrophil isolation and labelling existed at

the time of study, most were time consuming, requiring initial red cell sedimentation and preparation of a discontinous density gradient.

I developed a method where neutrophils were isolated from whole blood using a single-step procedure using Mono-Poly Resolving Medium (a Ficoll-Hypaque mixture). This circumvented the need to perform an initial red cell sedimentation step using dextran, hydroxyethyl starch or methylcellulose (Segal **et al**, 1978; Danpure **et** al, 1982; Pfieffer **et al**, 1982) as was current practice.

A "pure" cell population was isolated with a minimum of 'handling' and could be completed within one hour of venesection. The efficiency of cell separation was high, as was the purity of the sample, determined from differential leucocyte counts.

Advantages of neutrophil isolation using this method included preservation of cell viability by minimising manipulation of the cells. Also, when used in conjunction with non-discriminant radionuclides, ^{111}Indium oxine in this case, reduction in non-specific labelling of other blood cells, in particular avoidance of labelling radiosensitive lymphocytes was possible. Labelling efficiency of 70-80% of the pure cell isolate agreed well with other published data (Thakur **et al**, 1977; Mountford **et al**, 1985).

Since my work was completed, alternative means of neutrophil labelling have been demonstrated. Two of the major developments is that of ^{99m}Technetium, which is used to label mixed leucocyte populations and constitutes a cheap and readily available source of radioactivity. Secondly, specific labelling of neutrophils in vitro or in vivo with monoclonal antibodies to neutrophil surface glycoproteins.

Technetium labelling has been tried before but the instability of ^{99m}Tc in combination with ligands thwarted these attempts. However the

re-discovery of an agent, already used as a brain scanning agent, hexamethylpropylene amine oxime (HMPAO), made ^{99m}Tc labelling feasible (Ell **et al**, 1985).

Several studies report the successful use of mixed leucocyte populations labelled with 99mTc-HMPAO in the study of inflammatory lesions, with detection as early as 4 to 6 hours after injection (Peters **et al**, 1986; Roddie **et al**, 1988). However the relatively short half life of ^{99m}Tc (6.02 hours), may be a problem as maximum white cell localisation may not take place for up to 24 hours.

While initial reports are promising, there may be other associated limitations of its use. For example, reduced efficacy was demonstrated by studies in dogs where significantly lower accumulation of ^{99m}Tc-HMPAO activity located in abscesses compared with simultaneously injected ^{111}Indium-labelled leucocytes (McAfee **et al**, 1987) was found. Also higher radiation doses are routinely administered due to the considerably shorter half-life of ^{99m}Technetium. A radiation dose of around 740 rads (0.5mCi or 18.5Bq) is generally associated with indium-labelled leucocytes whereas for ^{99m}Tc a dose of 3120 rads is common. Whilst it was previously thought that administration of 10 000 rads and below had no effect on cell function, more recent studies suggest that this may be a considerable over-estimate. This was illustrated when cells, exposed to a 60-Cobalt gamma cell, showed marked impairment of localisation at sites of inflammation (Bassano **et al**, 1979). Also such high doses may potentiate the risks associated with radiolabelling lymphocytes, since this radionuclide is recommended for use with mixed leucocyte populations.

The second approach, designed to circumvent the complexities of labelling leucocytes in vitro, is the development of methods for

labelling neutrophils in vivo using monoclonal antibodies. These monoclonals, raised against cell membrane glycoproteins, may be labelled with ^{99m}Tc or ^{111}In, which permits imaging as a detection system.

This relatively new approach has several disadvantages including uncertainty on the exact mode of action (Gardner **et al**, 1989), the possibility of reactions to the murine antibodies (Seybold **et al**, 1988) and of more consequence to this type of study, that granulocyte-associated radioactivity accounts for less than 30% of the injected dose (Joseph **et al**, 1988).

While possible refinements of these methods may eventually lead to the successful use of these agents in vivo, cells isolated on M-PRM and subsequently labelled with ^{111}Indium-oxine provided the optimal means of studying the specific nature of neutrophil involvement in acute myocardial infarction.

The effects of isolation procedures; the isolation media used, centrifugation steps, temperature and completion time, on neutrophil function remains a contentious issue.

While assessment of viability was carried out on the cell isolates using the trypan blue exclusion test, this was a crude measure of the cell's state. Results from studies on the effects of Ficoll-Hypaque and Percoll, on neutrophil activation, as assessed by release neutrophil elastase, a recognised marker of cell activation (Greer **et al**, 1989) showed neither agent caused significant cell activation. More notably, agents used to effect red cell sedimentation were shown to represent a more important source of neutrophil activation with the compounding effect of increased temperature also clearly illustrated. Therefore

where 'pure' neutrophil isolates are required, a one step isolation procedure, avoiding the requirement for use of sedimentation agents, provides the best option.

A common technique of assessing cell viability of neutrophils after labelling is their passage, after intravenous injection, through the lungs and subsequent collection in the liver and spleen. Ideally, leucocyte transit should be compared to that of the red cell population, generally labelled with ^{99m}Tc (Muir **et al**, 1984). This method requires simultaneous acquisition of both isotopes but because of data processing limitations was beyond the scope of our equipment. In this study a single-isotope method was used to compare the transit of neutrophils through the lungs with the heart.

Several studies have shown that poorly functioning or damaged ^{111}Indium labelled neutrophils demonstrate lung retention or liver sequestration (Weiblen **et al**, 1979; Saverymuttu **et al**, 1983). Indeed there has been considerable debate about the chemical form of indium with at least one group favouring troplone over indium. Isolated granulocytes, labelled with ^{111}Indium tropolone in plasma were shown to exhibit "rapid transit through the pulmonary vasculature" whereas cells labelled in saline exhibited behaviour that represented "stimulation and/or damage" (Saverymuttu **et al**, 1983). Because ^{111}Indium oxine will preferentially label the proteins in plasma (McAfee, 1984) , neutrophils must be labelled in saline, as was the case in this study.

The cells isolated on M-PRM and labelled with ^{111}indium oxine followed "typical" in vivo behaviour, in that lung retention of the cells was minimal, with subsequent accumulation in liver and spleen, in

a manner similar to previous observations (Muir **et al**, 1984).

Thus pure neutrophils separated and labelled as described have good in vivo kinetics and appear functionally normal.

"The ultimate test of granulocyte function from a clinical point of view" according to Joseph **et al**, (1988), is that "they accumulate in abscesses and other inflammatory lesions".

Such a quality was demonstrated for the cells used in this study. In those patients, considered here in isolation from those with acute myocardial infarction, indium activity, representing neutrophil uptake was shown in patients in whom active infection of inflammation was later confirmed.

The frequency of the positive images here was relatively low but probably reflects the large number of requests for this test as a means of diagnosis in cases where other diagnostic tests had proved inconclusive.

Failure to detect an infection site occurred in only one subject. This case of a subdiaphragmatic abscess was later found at post mortem and presumbly was undetected due to the proximity of liver and spleen, both normally sequestering labelled neutrophils.

Leucocyte scanning proved especially useful in diagnosing infection of inflammatory foci in the bowel. This method has advantages in that, unlike ^{67}Gallium and ^{99m}Tc-HMPAO, background and non-specific uptake radioactivity in the gut does not interfere with image interpretation (Costa **et al**, 1988).

Detection of neutrophil uptake was shown in a case of pneumonia and contrasts to results of a study by Lavender **et al**, (1988). While diagnosis is normally made using routine radiological techniques, the

inability to do so with labelled leucocytes has puzzled many groups working in this field. This was originally attributed to reduced lung perfusion during the disease process, but has since been disproved (Lavender **et al**, 1988). Successful imaging found here was thought to relate to the timing of the study. Autologous labelled neutrophils were administered very early in the progress of the pneumonia and preceded commencement of antibiotic therapy. This would suggest that the stimulus for neutrophil recruitment to the site of inflammation may be early and somewhat transient. This postulate was developed further in the study of factors which determined neutrophil uptake in the heart after myocardial infarction.

This study confirmed that using labelled autologous neutrophils, the inflammatory response to acute myocardial infarction could be imaged in man.

In 23 of the 30 patients studied, uptake of indium activity in the region of the heart, was demonstrated by planar or SPET imaging or a combination of both. Factors that were considered might influence the outcome of imaging were assessed. The only determinant that could reliably be used to predict the outcome of imaging was the time span between the onset of the major chest pain and injection of the labelled cells. The earlier after the event the cells were injected, the greater the probability of obtaining a positive image, a fact clearly demonstrated by the 100% success rate in patients reinjected within 18 of infarction which fell dramatically to 44% if delayed to between 24 and 36 hours.

The temporal relationship found here suggests that the stimulus for neutrophil recruitment into the injured myocardium is an early and

somewhat transient phenomenon. As explained above, the concept of a "time-window" for cell migration may also explain why some workers were unable to demonstrate neutrophil uptake in pneumonia. If labelled cells are administered late in the disease process, the signal or stimulus for neutrophil migration may be "switched off" (Lavender **et al**, 1988).

The timing of the sequence of events, particularly that relating to early maximal cellular involvement, may appear to conflict with evidence from pathologic studies where maximal neutrophil presence is seen 2 to 4 days after infarction (Mallory **et al**, 1939). However it must be noted that pathological observations only show the total accumulation of neutrophils and do not address the dynamics of the process.

Information from planar images is often limited. Interference from physiological activity located in spleen and liver makes it difficult to unequivocally demonstrate uptake in specific locations. Improved anatomical localisation was achieved with the use of single photon emission computed tomography (SPET). Further improvement of activity localisation was obtained by administration of 99mTc-HSA, a blood pool marker which allowed differentiation between tissue or blood pool associated activity.

The use of SPET resulted in significant improvement in anatomical localisation of indium uptake in the earlier studies of inflammation/infection. This was important in the 2 patients who sustained a perforated bowel and mycotic aneurysm repectively. In the latter case, further definition was achieved employing SPET in conjunction with a blood pool marker (^{99m}Tc-human serum albumin). More importantly, the use of SPET in the study of myocardial infarction also

increased the number of positive images found by making separation of the inferior surface of the heart from liver and spleen possible .

The results from the imaging studies show neutrophil infiltration to be a relatively early phenomenon in acute myocardial infarction.

The second phase of the work set out to examine evidence of neutrophil activation.

It has been known for some time that the leucocyte count is increased in ischaemic heart disease (Ernst **et al**, 1987; Kostis **et al**, 1984). An association between leucocyte count and myocardial infarction, particularly prediction of first and subsequent re-infarction, has also been established (Friedman **et al**, 1974; Lowe **et al**, 1985). Certainly my own observations again demonstrate this increase in leucocyte count in patients with ischaemic heart disease.

The leucocyte count was also elevated in the patients with acute myocardial infarction (Nash **et al**, 1989), but reflects the more general response to stress (Muir **et al**, 1984).

In view of the contribution the neutrophil makes to the rheology of the blood, and its interaction with the vascular endothelium, it is believed the presence of high numbers of neutrophils or perhaps activated neutrophils, may contribute to the risk of ischaemic events by promoting vascular occlusion, post occlusive inflammatory injury and endothelial injury (Dinerman **et al**, 1990).

To establish whether the increased leucocyte numbers in these groups represented an activated cell population, markers of in vivo neutrophil activation were also measured.

Such measurement, in plasma, of substances released from neutrophil granules provides a means of assessing cell activation in vivo (Ernst **et al**, 1987). Neutrophil elastase, a neutral protease contained in the azurophilic granules, was measured in plasma using a standard radioimmunoassay technique. The polyclonal antisera raised in-house, against human neutrophil elastase, was specific, in that it did not bind to the antigenically distinct elastases of platelets and pancreas. It detected neutrophil elastase in its free and also in complex with its natural inhibitors, the state in which it circulates in plasma.

When measured in whole blood, elastase correlated with the total neutrophil count and is primarily a measure of the intracellular stores of this protease. Neutrophil elastase in plasma does not correlate with leucocyte count or neutrophil count and therefore the elevated levels reflect an increase in neutrophil activation in the form of degranulation (Greer **et al**, 1989).

Plasma neutrophil elastase (PNE) was elevated significantly in patients with stable ischaemic heart disease, and although neutrophil count was raised in this group, no correlation with plasma neutrophil elastase was found and therefore was consistent with a prevailing state of neutrophil activation.

In the patients with acute myocardial infarction, neutrophil elastase was raised over the 48 hour period following infarction and was further raised at around 24 hours. This peak of activity coincided with a time when the inflammatory infiltrate is known to be present histologically in the myocardium, as shown by the imaging studies.

In a similar study of patients with unstable angina and acute myocardial infarction, evidence of neutrophil activation was found (Mehta **et al**, 1988). In contrast however, no evidence of in vivo

neutrophil activation was found in patients with stable ischaemic heart disease, although a state of "hyperactivity" was indicated by increased neutrophil function. Therefore evidence is emerging to indicate the existence of neutrophil activation in ischaemic heart disease. This enhanced activity, as seen in these patients, therefore may provide a pathophysiologic environment for progression from stable to unstable coronary heart disease.

An association between in vivo neutrophil activation and vascular disease have been reported. Several of these studies, carried out in Edinburgh on diabetes mellitus (Collier **et al**, 1989), pregnancy-induced hypertension (Greer **et al**, 1989) and groups particularly at risk from vascular disease (Jackson **et al**, 1991) have shown evidence of neutrophils contributing to the progession of the disease. Although attempts to correlate severity of disease with elastase levels have proved inconclusive, where end-organ damage was present, circulating plasma levels of elastase were further increased.

The non-peroxide isomer of linoleic acid, PL-9,11-LA', was used as a indicator of free radical activity. The method used was a modification of that of Iversen **et al**, (1985) using high performance liquid chromatography.

PL-9,11-LA', indicating free radical activity, was raised in the patients with acute myocardial infarction, but not in patients with ischaemic heart disease and was consistent with the production of free radical activity after myocardial infarction.

In the early part of this study only the PL-9,11-LA' isomer was measured. In view of concern on the origins of linoleic acid in vivo,

especially through dietary intake, in subsequent studies, the substrate, linoleic acid (PL-9,12-LA) was also measured. In this way the molar ratio of the isomer to its substrate could be expressed, thus providing a more sensitive index of change.

However in the patients with acute myocardial infarction PL-9,11-LA' levels were measured sequentially over a period of 48 hours and therefore changes associated with dietary intake over this period of hospitalisation would seem unlikely.

As yet electron spin resonance spectroscopy (ESR), represents the best method for detecting free radical activity in vivo. However as discussed in Chapter 1, this system, while readily applicable to the chemistry of non-biological systems, its use in living systems presents significantly greater problems, although it has been used with some success in animal models. Once this method has been modified for more general in vivo use, valuable information, most specifically on the site of production may be provided.

Until this is possible, indirect methods of assessment must suffice. It may be prudent then, where feasible, to use a range of indicators of free radical activity to generate a more comprehensive picture of these processes. This should include measurement, not only of body constituents altered as a result of free radical interaction but also scavengers and anti-oxidants. Several recent studies have measured at least four indirect indicators of free radical activity eg. superoxide dismutase, plasma thiol, erythrocyte lysate thiol and caeruloplasmin (Collier **et al**, 1990). Also detection of anti-oxidants, such as vitamins E,C, A and also carotene (Reimersma **et al**, 1991) may provide further

information.

As discussed in Chapter 1, evidence of infarct size attenuation by neutrophil depletion in animals, suggests that neutrophils may cause secondary heterolytic damage of myocytes after myocardial infarction (Romson **et al**, 1982; Jolly **et al**, 1986). This additional damage was attributed to the release of lysosomal enzymes (Engler **et al**, 1987) or to free radical production (McCord, 1985). Such damage is thought to be further augmented by institution of reperfusion by intravenous thrombolysis (Braunwald **et al**, 1985). This additional damage, termed "reperfusion injury" is thought to off-set the known benefits of reperfusion (ISIS-2, 1988; GISSI-2, 1990) by mechanisms of injury including capillary plugging (Kloner **et al**, 1974), generation of oxygen-derived free radicals (Werns **et al**, 1986) and neutrophil activation with concomitant release of lysosomal enzymes (McCord, 1987). As a result of these studies in animals, possible therapeutic intervention to reduce or abrogate neutrophil recruitment and activation during myocardial ischaemia has been suggested (Crawford **et al**, 1988).

Since the completion of this study, several investigators have used indirect methods of assessing free radical production in man, after periods of induced clinical ischaemia. Coronary angioplasty (Roberts **et al**, 1990), pacing (Oldroyd **et al**, 1990) and coronary bypass surgery (Davies **et al**, 1990) provided models of ischaemia and reperfusion. Each of these studies detected free radical activity by measurement of lipid peroxidation products and sampled almost upon the point of reperfusion.

While there are variations in the results, the general finding was that there is a small, but significant increase in free radical production within minutes of reperfusion. A study of free radical production in man after thrombolysis by Davies **et al**, (1990), showed that successful thrombolysis was associated with increased lipid peroxidation two hours after administration.

The data from Chapter 7 had shown that the acute inflammatory response to myocardial infarction could be imaged in man and the studies in Chapter 9 confirmed this. Moreover the observation from Chapter 8 that there is evidence of neutrophil activation and free radical activity was also confirmed in Chapter 9. Reperfusion injury has been demonstrated in animals but when we examined patients treated with intravenous thrombolysis, there was no evidence of enhanced neutrophil activation and free radical activity.

Animal models suggest that neutrophil activation and free radical activity can extend myocardial injury. Our own observations confirm that neutrophil activation and free radical activity occurs in man following infarction, but we can make no observations on how this affects infarct size. At present therefore there seems little justification for the use of anti-inflammatory drugs in man, particularly since, while known to reduce ischaemic myocardial injury in animals, these may adversely affect the reparative process, resulting in subsequent scar thinning (Cannon **et al**, 1985; Jugdutt **et al**, 1985).

Clearly much remains to be done. Improvements in radiolabelling techniques continue, making more detailed examination of the in vivo behaviour of the neutrophil possible. Other markers of neutrophil

activation exist and elastase is certainly not the only proteolytic enzyme released by the neutrophil. A more detailed examination of the mechanisms of secondary destruction of the myocyte is essential. Improved methods of studying the role of reactive oxygen are required and perhaps ESR will one day allow in vivo imaging of tissue release.

Given the practical and ethical difficulties of studies in man, it may continue to be difficult to assess the role of the neutrophil in myocardial damage in man. However the increasing importance of early intervention with revasularisation after acute myocardial infarction makes it all the more needy for means of documenting heterolytic damage to the myocardium.

Clearly our observations do not give cause for concern, as patients receiving thrombolysis seemed to sustain no additional damage. However the possibilities of further limiting infarct size and perhaps favourably affecting tissue repair make further observations of neutrophil behaviour in this common condition mandatory.

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Inflammatory response, neutrophil activation, and free radical production after acute myocardial infarction: effect of thrombolytic treatment

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Inflammatory response, neutrophil activation, and free radical production after acute myocardial infarction: effect of thrombolytic treatment

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Abstract

Activated neutrophils releasing proteolytic enzymes and oxygen free radicals have been implicated in extending myocardial injury after myocardial infarction. Neutrophil elastase was used as a marker of neutrophil activation and the non-peroxide diene conjugate of linoleic acid was used as an indicator of free radical activity in 32 patients after acute myocardial infarction; 17 were treated by intravenous thrombolysis. Patients with acute myocardial infarction had higher plasma concentrations of neutrophil elastase and the non-peroxide diene conjugated isomer of linoleic acid than normal volunteers or patients with stable ischaemic heart disease. Patients treated by thrombolysis had an early peak of neutrophil elastase at eight hours while those who had not been treated by thrombolysis showed a later peak 40 hours after infarction. The plasma concentration of non-peroxide conjugated diene of linoleic acid was highest 16 hours after the infarction irrespective of treatment by thrombolysis. Quantitative imaging with single photon emission tomography showed decreased uptake of indium-111 labelled neutrophils in the infarcted myocardium (as judged from technetium-99m pyrophosphate) in those who had received thrombolysis, suggesting a decreased inflammatory response.

The results indicate increased neutrophil activation and free radical production after myocardial infarction; they also suggest that thrombolysis does not amplify the inflammatory response and may indeed suppress it.

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Activation of neutrophils with release of lysosomal enzymes and production of oxygen free radicals is an important part of the host defence mechanism against microbial infection. Activated neutrophils, however, have been implicated in the pathogenesis of several disease processes including emphysema, adult respiratory distress syndrome, and myocardial infarction.[1] It has been suggested that the neutrophil may cause secondary heterolytic damage of myocytes, because neutrophil depletion limited infarct size in animal models of infarction[2 3]; the damage was attributed to the release of lysosomal enzymes[4] or free radical production.[5] Further experimental evidence suggests that reperfusion of ischaemic myocardium, for example after thrombolytic treatment, may be a double edged sword with the benefits of reoxygenation being partly offset by the potential harmful effects of reperfusion injury.[6] Postulated mechanisms of reperfusion injury include plugging of small capillaries,[7] generation of oxygen free radicals,[8] calcium influx[9] and neutrophil activation with release of lysosomal enzymes.[10] Thus the neutrophil may have a role in reperfusion injury but its importance in patients has not been determined, though the clinical benefits of thrombolytic treatment are now established.[11 12]

Degranulation of neutrophils releases neutrophil elastase, a lysosomal enzyme; this has been used as a specific marker of neutrophil activation.[13] Activated neutrophils are one potential source of oxygen free radical production.[14] It is difficult to measure these unstable oxygen species but once they are generated they will react and oxidise adjacent molecules, particularly polyunsaturated fatty acids.[15] The diene conjugated non-peroxide isomer of linoleic acid (phospholipid 9, 11-linoleic acid; PL-9, 11-LA') has been used as a marker of human free radical activity.[16] To determine if and when neutrophil activation takes place after myocardial infarction, we measured serial changes in plasma neutrophil elastase and PL-9, 11-LA'. We also investigated how these indices were influenced by reperfusion by studying patients treated with and without thrombolytic agents. Finally, because the acute inflammatory infiltrate within myocardium after myocardial infarction can be imaged,[17] we examined the effect of treatment with and without thrombolysis on the myocardial uptake of indium-111 labelled neutrophils.

Patients and methods

VALUES IN HEALTHY VOLUNTEERS AND PATIENTS WITH CHRONIC ISCHAEMIC HEART DISEASE

To establish a normal range for plasma concentrations of neutrophil elastase and the conjugated diene of linoleic acid, blood samples were taken from 35 healthy volunteers from laboratory and hospital staff. As a further control group we also studied 30 patients with a documented history of ischaemic heart disease based on coronary angiography (n = 12) or distant myocardial infarction (n = 18) (table 1).

PATIENTS WITH ACUTE MYOCARDIAL INFARCTION

Observations were based on 32 patients who

Table 1 Characteristics of healthy volunteers, patients with chronic ischaemic heart disease, and patients with myocardial infarction

			Myocardial infarction	
	Volunteers (n = 35)	*IHD (n = 30)*	*No thrombolysis (n = 15)*	*Thrombolysis (n = 17)*
Age (yr)	34 (22–63)	59 (37–76)	58 (38–74)	56 (30–69)
M/F	30:5	24:6	10:5	13:4
WBC ($\times 10^9$/l)	5·8 (3·4–9·3)	6·7 (5·2–12·6)	13·2 (9·6–23·0)	16·8 (7·9–33·7)
LVEF (%)	—	41 (18–63)	32 (15–71)	40 (24–68)
Peak creatine kinase (U/l)	—	—	1635 (522–6255)	2059 (533–6955)
Heparin (subcutaneously)	—	—	15	—
Heparin (intravenously)	—	—	—	17
Streptokinase/anistreplase	—	—	—	9/8
Lignocaine	—	—	3	5
Hydrocortisone	—	—	—	9
β Blockers	—	24	2	5
Diuretics	—	4	6	5
Deaths	—	—	2	1

WBC, white blood cells; LVEF, left ventricular ejection fraction.

had sustained a recent acute anterior myocardial infarction. The diagnosis was based on the history of prolonged ischaemic chest pain, characteristic electrocardiographic changes, and increase in the enzyme creatine kinase. The patients were subdivided into the 17 who had been given intravenous thrombolysis with either 1 200 000 units streptokinase (Kabivitrum, Middlesex, UK), or 30 units of anistreplase (anisoylated plasminogen streptokinase complex (Eminase, Beecham Pharmaceuticals, Epsom, UK). The other 15 were deemed ineligible to receive thrombolytic treatment because of late admission (> 4 hours after the onset of symptoms), a history of active peptic ulceration, other source of haemorrhage, or recent cerebrovascular accident and were treated in conventional fashion. Table 1 shows the details of the two groups including drug treatment. All study times were taken from the onset of symptoms. All patients gave informed consent and the study was approved by the institute's ethics committee.

PROTOCOL

In patients with acute myocardial infarction blood was taken for full blood count and measurement of creatine kinase, neutrophil elastase, and linoleic acid (PL-9, 12-LA) and its diene conjugated non-peroxide isomer (PL-9, 11-LA′) esterified as phospholipids. Samples were taken at 6–8 hours after the onset of symptoms and every 8 hours thereafter until 48 hours. Samples were centrifuged and plasma separated immediately and held at −20°C until analysis was performed.

PL-9, 11-LA′ AND PL-9, 12-LA

The molar concentrations of PL-9, 11-LA′ and PL-9, 12-LA in plasma were measured by high performance liquid chromatography in plasma after enzymatic hydrolysis with phospholipase A_2 and solid phase sample preparation as described by Iversen *et al.*[16] The intra-assay coefficient of variation was < 3·5%. The results are expressed as μmol/l.

PLASMA NEUTROPHIL ELASTASE

Human neutrophil elastase was measured by specific radioimmunoassay with rabbit polyclonal antiserum.[13] The antigen was purified from human neutrophils after leucopheresis. The antibody was specific for neutrophil elastase and measured free enzyme and enzyme complexed to the natural inhibitors α_1-proteinase inhibitor and α_2-macroglobulin equally well. The results are expressed as ng/ml and the intra-assay coefficient of variation was < 5%.

IMAGING

Our previous work has shown that labelled neutrophil uptake within the myocardium can be imaged reliably providing the time from onset of infarction to reinjection of labelled cells is < 18 hours.[17] Only 21 of the patients could be studied by this technique within the appropriate time limits because of limited availability of the radionuclide indium-111. The patients received 20–30 MBq of indium-111 labelled autologous neutrophils within 18 hours of the onset of symptoms. Single photon emission tomography was undertaken 24 hours after the injection of the labelled cells with an IGE 400 AT maxicamera linked to a Siemens Microdelta computer. On the following day the patients were injected with 500 MBq of (technetium-99m labelled pyrophosphate (^{99m}Tc-PYP) and two hours later further SPET imaging was undertaken to estimate infarct size. Apart from the different energy windows and imaging time the protocol on each day was identical. The single photon emission tomographic imaging was performed over 360°, 64 images being acquired and reconstruction being undertaken by back projection with a Butterworth filter to create sagittal, coronal, and transverse images. A study was regarded as positive when uptake could be clearly seen in all three views. In these studies all transverse slices for both indium-111 and ^{99m}Tc-PYP with uptake in the region of myocardium were then analysed by a semiautomatic programme that counted the number of volume cell elements (voxels) with values greater than 65% of the peak myocardial uptake.[18] For each patient it was possible to compare the volume of myocardium showing neutrophil uptake with the volume of infarcted myocardium as judged from the pyrophosphate images.

Patients also had a predischarge radionuclide

ventriculogram to assess residual left ventricular function.

STATISTICAL ANALYSIS

The Kolmogorov-Smirnov test showed that the data were not normally distributed. Results are therefore expressed as median and range. We used non-parametric analysis (Wilcoxon rank sum test) for two independent samples (Mann-Whitney). Values of $p < 0.05$ were taken as significant and data were analysed by computer with the statistical package for social sciences (SPSS Inc, Chicago).

Results

WHITE BLOOD CELL COUNT

The white blood cell count was significantly lower in the normal volunteers (5.8×10^9/l, 3.4–9.3/l) than in patients with ischaemic heart disease without recent infarction (6.7×10^9/l, 5.2–12.6/l, $p < 0.004$) and in both groups counts were lower than the initial white cell count in patients with acute myocardial infarction (16.4×10^9/l, 7.9–33.7, $p < 0.0001$). This was also true for the neutrophil count for the three groups.

PLASMA NEUTROPHIL ELASTASE

The plasma concentration of neutrophil elastase was significantly lower in the normal volunteers (18.6 ng/ml, 9.2–51.0) than in the patients with stable ischaemic heart disease (25.8 ng/ml, 12.2–49.5, $p < 0.002$). For the 48 hours after myocardial infarction, however, plasma neutrophil elastase was higher than in either normal volunteers or patients with ischaemic heart disease (fig 1a). The time course differed in patients who had received thrombolytic treatment and in those who had not.

PL-9, 11-LA′ AND THE MOLAR RATIO PL-9, 11-LA′/PL-9, 12-LA

There was no significant difference in PL-9, 11-LA′ or the molar ratio PL-9, 11-LA′/PL-9, 12-LA between normal volunteers (19.3 μmol/l, 7.5–32.9; 4.7, 1.9–9.1) and patients with ischaemic heart disease (19.8 μmol/l, 7.9–43.2; 5.4, 1.7–12.1). For the 48 hours after myocardial infarction PL-9, 11-LA′ was significantly greater than in the healthy volunteers. The concentration reached a maximum at 16 hours; by 48 hours the values were not significantly greater than those of patients with chronic ischaemic heart disease (fig 1b). The molar ratio of PL-9, 11-LA′/PL-9, 12-LA was significantly increased in the 48 hours after myocardial infarction and followed a similar time course.

COMPARISON OF PATIENTS WITH MYOCARDIAL INFARCTION WHO WERE TREATED BY THROMBOLYSIS AND THOSE WHO WERE NOT

There was no significant difference in peak creatine kinase activity between patients treated conventionally and those who were treated by thrombolysis. Plasma activity of creatine kinase reached a peak earlier in those treated by thrombolysis (16 hours) than in those treated conventionally (24 hours). Similarly, there was no significant difference in the left ventricular ejection fraction measured by radionuclide ventriculography, 10 days after infarction, though those patients who were treated by thrombolysis had slightly higher values (table 1).

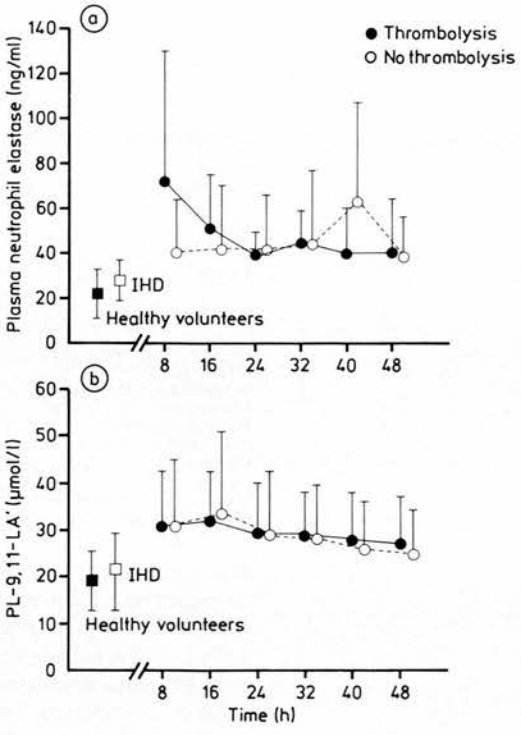

Figure 1 (a) Mean plasma neutrophil elastase (1 SD) in healthy volunteers, patients with chronic ischaemic heart disease (IHD), and those with acute myocardial infarction. Changes in plasma concentration neutrophil elastase (PNE) after myocardial infarction are shown for those treated with and without thrombolysis. The values of PNE are significantly greater at all sampling times after myocardial infarction than in healthy volunteers or patients with chronic ischaemic heart disease. Patients treated by thrombolysis have significantly higher values of PNE at 8 hours ($p < 0.025$) and lower values at 40 hours ($p < 0.037$) than those not treated by thrombolysis. (b) Mean PL-9, 11-LA′ (1 SD) in the same groups showing significantly higher values after myocardial infarction than in ischaemic heart disease or in healthy volunteers. There was no difference between those treated by thrombolysis and those not treated by thrombolysis at any time.

PLASMA CONCENTRATIONS OF NEUTROPHIL ELASTASE

The pattern of change in plasma neutrophil elastase differed in the two groups of patients with acute myocardial infarction (fig 1a). Patients given intravenous thrombolysis had higher early maximal values at 8 hours (48.2 ng/ml, 25–250) than those treated conventionally (32.6 ng/ml, 15.6–101, $p < 0.025$). Those treated conventionally tended to have lower early values with a late peak at 40 hours (49.8 ng/ml, 21.4–196) than those treated with thrombolysis (34.2 ng/ml, 15.8–83, $p < 0.037$).

PL-9, 11-LA′ AND THE MOLAR RATIO PL-9, 11-LA′/PL-9, 12-LA

There was no significant difference in PL-9, 11-LA′ or the molar ratio PL-9, 11-LA′/PL-9, 12-LA in those treated conventionally after

Table 2 Details of patients treated with and without thrombolysis who were imaged with ^{111}In labelled neutrophils and ^{99m}Tc pyrophosphate

	CK max (U/l)	*LVEF (%)*	*Time to injection (h)*	*^{111}In (voxels)*	*^{99m}Tc (voxels)*	*$^{111}In/^{99m}Tc$*
No thrombolysis (n = 10)	2508 (522–6255)	36 (15–51)	8 6·5–10	114 (19–276)	201 (77–405)	0·79 (0·06–2·14)
Thromolysis (n = 11)	2500 (533–6955)	40 (27–68)	11 5–18	81 (0–160)	217 (111–323)	0·41 (0–0·96)
p	NS	NS	NS	NS	NS	<0·05

(Each voxel or volume picture cell element represents approximately 250 mm^3.)
CK, creatine kinase; LVEF, left ventricular ejection fraction.

myocardial infarction and those given thrombolysis. For both groups the values were greatest at 16 hours, gradually falling towards normal values (fig 1b).

There was no correlation between the white blood cell count and plasma concentration of neutrophil elastase in any of the groups. There was a weak correlation between white cell count and PL-9, 11-LA′ in the non-thrombolytic group ($r = 0·63$, $p < 0·02$), and in patients with ischaemic heart disease ($r = 0·45$, $p < 0·02$), but this did not hold for the corrected molar ratio and no correlations were found in the normal volunteers and patients treated with thrombolysis.

IMAGING (TABLE 2)

In general, in the patients who were imaged there was a significantly greater number of voxels showing an uptake of ^{99m}Tc pyrophosphate than of ^{111}In labelled neutrophils ($p < 0·0006$). Uptake of ^{99m}Tc pyrophosphate was very similar in patients who were treated by thrombolysis and those who were not, suggesting little difference in infarct size in these patients. This is supported by similar values of residual left ventricular ejection fraction and peak creatinine kinase in both groups (table 2). In contrast, the uptake of ^{111}In labelled neutrophils was less in the patients who were treated by thrombolysis. Thus the ratio of $^{111}In/^{99m}Tc$, which serves as an estimate of the inflammatory response for a given infarct size, was significantly less in patients treated by thrombolysis (0·41, range 0–0·96) than in those treated without (0·79, range 0·06–2·14, $p < 0·05$). In fig 2 an example of the greater uptake of ^{111}In labelled neutrophils in a patient treated without thrombolysis is contrasted with that seen in a patient who had received streptokinase.

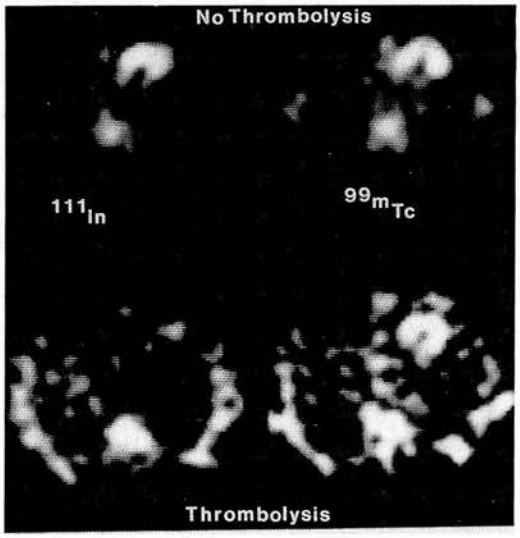

Figure 2 Single photon emission tomographic images comparing the uptake of ^{111}In labelled neutrophils with the size of infarction as assessed by ^{99m}Tc-pyrophosphate (PYP). The upper pair of images show easily detected uptake of ^{111}In and ^{99m}Tc-PYP in a patient with anterior myocardial infarction who was not given thrombolytic treatment. The lower pair of images show markedly reduced uptake of ^{111}In labelled neutrophils compared with ^{99m}Tc-PYP in a patient with anterior myocardial infarction treated with anistreplase.

Discussion

We found both an increased neutrophil elastase release and an increase in the plasma concentrations of the diene conjugated non-peroxide isomer of linoleic acid (PL-9, 11-LA′) after myocardial infarction. The association of leucocytosis and the severity of myocardial infarction was first described by White in 1926.[19] We noted increased neutrophil leucocytosis after myocardial infarction but there was no correlation between the peripheral neutrophil count and the raised plasma concentrations of neutrophil elastase. A distinction must be made between whole blood elastase and plasma elastase. Whole blood elastase correlates with the total neutrophil count but is not a marker of neutrophil activation and reflects the much greater intracellular stores of the enzyme. Increased plasma neutrophil elastase relates to neutrophil degranulation and presumably reflects release within the area of myocardial injury. The peripheral leucocytosis is part of the more general response to stress.[20] Like others we also noted an increased leucocyte count in patients with stable ischaemic heart disease and this has been shown to be a predictor of future myocardial events.[21] The neutrophil has been recognised as an early part of the inflammatory response to myocardial infarction,[22 23] but it is only more recently that experimental evidence has been put forward to suggest that it may be involved in secondary heterolytic myocyte damage after myocardial infarction[4 5] and that suppression of the neutrophil infiltrate can reduce infarct size.[2 3] One mechanism by which activated neutrophils can induce cell damage is through the release of potent proteolytic enzymes.

Neutrophil elastase is a serine protease released from the primary granules after major cell stimulation such as phagocytosis or cell death.[24] It has a wide range of substances including elastin, collagen, fibrinogen, and other matrix macromolecules and is implicated in the pathogenesis of several human diseases.[1] Our study showed that plasma concentrations of human neutrophil elastase were significantly higher in patients after myocardial infarction than in healthy volunteers and patients with stable ischaemic heart disease. Further, the pattern of elastase release was different in patients treated conventionally and in those treated with thrombolysis. In patients treated with streptokinase or anistreplase there was a significant early increase in plasma neutrophil

elastase within the first few hours of treatment. This confirms previous observations[25] and it seems likely that this represents clot lysis or intracoronary activation of neutrophils because few inflammatory cells will have migrated into myocardial tissue by this stage.[22 23]

In contrast, in patients treated conventionally concentrations of neutrophil elastase reach a peak later, between 32 and 40 hours after the onset of symptoms, when the inflammatory infiltrate is present histologically and can be imaged.[17] Drug treatment, particularly hydrocortisone or lignocaine, might be expected to influence neutrophil behaviour. But a similar number of patients in both groups received lignocaine, which is known to depress neutrophil function in vitro.[26] Also patients given hydrocortisone before streptokinase did not have lower ^{111}In uptake by neutrophils or lower concentrations of elastase than patients treated with anistreplase, who did not receive hydrocortisone. This suggests that the results in the thrombolytic group are not influenced by the potential suppressive effect of hydrocortisone on the inflammatory response and neutrophil function.[27]

Although the neutrophil is a source of cytotoxic oxygen species, there are other potential sources of oxygen free radical production after myocardial infarction, including the conversion of xanthine dehydrogenase to xanthine oxidase, mitochondrial production, the auto-oxidation of catecholamines, and the arachidonic acid cascade.[8] Although xanthine dehydrogenase is converted to xanthine oxidase in the vascular endothelium, this enzyme system is not present in human myocytes and is therefore unlikely to be directly involved in myocardial damage.[28] Therefore, the most likely sources of free radical production within the human myocardium are the electron transport chains of the myocyte mitochondria or from activated neutrophils via the membrane linked nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH) oxidase.[10] Our studies of peripheral blood did not define the increase as being of myocardial origin, but the myocardium is the most likely site in patients who are haemodynamically stable.

Products of lipid peroxidation have been used as markers of free radical activity because exposure of cell membranes to oxygen free radicals stimulates "lipid peroxidation"[29]—perhaps better termed lipid oxidation. Lipid peroxidation products have themselves been implicated in damaging cell membranes after myocardial infarction,[30] though some have suggested that increased evidence of lipid peroxidation merely reflects cell damage.[15] For this reason we chose to measure the diene conjugated non-peroxide isomer of linoleic acid PL-9, 11-LA′ in plasma because this is thought to be a marker of human free radical activity.[16 31] PL-9, 11-LA′ is present in the diet and can be manufactured by bacterial flora and this may account for the conflicting reports of the usefulness of this as a marker of cervical cancer.[32 33] It is unlikely that changes in dietary intake or bacterial flora would alter significantly in the 48 hours after myocardial infarction to account for the changes seen in plasma PL-9, 11-LA′. Also the concentrations were not increased in the patients with stable, but documented ischaemic heart disease and there was no change in linoleic acid, which is also affected by dietary intake.

Heparin was shown to increase 9,11-LA′[34]—the assay method, which is designed to measure the molar concentration of 9,11-LA′and 9,12-LA esterified as phospholipids, also measures 9,11-LA′ and 9,12-LA present as free fatty acids. Free fatty acids are increased by the lipolytic action of heparin though the magnitude of the response is often overestimated because the method does not take account of the extensive ex-vivo lipolysis.[35] In our study heparin is unlikely to have had any significant effect because the thrombolytic group received only intravenous heparin as an adjuvant to thrombolysis a mean of 18 hours 36 minutes after the onset of symptoms. The patients treated conventionally also received heparin but as a low dose subcutaneously. We noted no difference in the values of PL-9, 11-LA′ between the two groups despite the widely different heparin regimens. The similarity of responses of the two groups also argues for a lack of effect of the thrombolytic drugs in themselves on PL-9, 11-LA′.

The concentration of PL-9, 11-LA′ or the molar ratio did not correlate with creatine kinase measured on the same sample and it therefore seems unlikely that the raised concentrations are merely a marker of cell damage[15] and the evidence is consistent with the diene being a free radical marker in human beings. In general, in patients with myocardial infarction the values of PL-9, 11-LA′ and the molar ratio were high initially and then fell; this suggests that maximum free radical generation occurs early. We were unable to detect any difference in the concentrations of PL-9, 11-LA′ or the molar ratio of PL-9, 11-LA′/PL-9, 12-LA between patients treated conventionally and those who received thrombolysis. This lack of difference in the molar ratio (indeed it was somewhat lower in the group treated by thrombolysis) suggests that reperfusion was not associated with a delayed or secondary increase in free radical production with the potential for further myocardial damage. Even when samples were taken earlier (less than 6 hours after the onset of symptoms) we did not detect any difference in those treated by thrombolysis. We cannot exclude the immediate increase in free radical production after reperfusion shown in some animal studies.[36] However, in human beings free radical injury after reperfusion may be reduced by the presence of red blood cells, which contain the antioxidant enzyme catalase[37] unlike the cell free perfusate often used in animal experiments.

This study confirms that the human inflammatory response to myocardial infarction can be imaged. In only two of the 21 patients studied no uptake of ^{111}In-labelled neutrophils could be detected. Both patients had been treated by thrombolysis and although this

result could represent failed imaging, this is unlikely because all the thrombolytic group had significantly reduced uptake of neutrophils in relation to infarct size assessed by ^{99m}Tc-pyrophosphate. The concept of a reduced inflammatory response is supported by the different patterns of release of neutrophil elastase. If the early neutrophil activation in those given thrombolysis represents intracoronary activation and the late response corresponds to acute inflammatory infiltrate within damaged myocardium we would expect greater neutrophil uptake in those who did not receive thrombolytic agents. As an extension, the lack of a late increase in lipid oxidation suggests that the neutrophil is not the main source of free radical production in myocardial infarction. By inference this implies that if there is later heterolytic myocardial injury and if it is mediated by neutrophils, it may be secondary to release of proteolytic enzymes rather than oxygen free radicals. The results do not necessarily contradict the experimental evidence that suggests that free radical scavengers reduce myocardial damage,[36] because there is evidence that superoxide dismutase may work in part by reducing neutrophil migration.[38] It is therefore possible that some of the beneficial effects of thrombolysis are the result of partial inhibition of the inflammatory response to myocardial injury, perhaps by reducing the degree or duration of production of neutrophil chemoattractants, such as complement.[39 40]

Our studies suggest that after acute myocardial infarction neutrophils are activated and free radicals are produced. Although animal studies suggest such activation can extend myocardial injury we have no evidence of this in human beings. However, thrombolysis and presumed reperfusion are not associated with amplification of the inflammatory response or prolonged free radical production in patients. Indeed these responses seem to be diminished and some of the beneficial effects of thrombolysis may be the result of down regulation of the acute inflammatory response.

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Intrathoracic mycotic aneurysm detected by indium-111 labelled autologous neutrophils with single photon emission computed tomography

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Scintigraphy using neutrophils labelled with indium-111 (^{111}In) has been shown to be a reliable method of detecting occult infection,[1] particularly within the abdominal cavity.[2,3] We report a case in which an intra-abdominal source of infection was suspected but a labelled neutrophil scan showed the site to be an intrathoracic mycotic aneurysm, which was subsequently confirmed at thoracotomy.

Case report

A 63 year old woman was admitted to hospital for the investigation of a discharging sinus in the left iliac fossa. Eighteen months previously a Hartmann's procedure had been performed for a peridiverticular abscess and one month before this a right aorto-femoral graft and femoro-femoral crossover graft had been performed for severe peripheral vascular disease. On admission the patient was febrile (38°C) and abdominal examination showed previous operative scars, with a discharging sinus in the left iliac fossa. The remainder of the clinical findings were entirely normal. Apart from a slightly raised leucocyte count (11·2 × 10^9/l) and a growth of *Staphylococcus aureus* from the sinus discharge fluid, routine investigations all gave normal results. A sinogram failed to show any communication between the sinus and the bowel. A right transfemoral angiogram

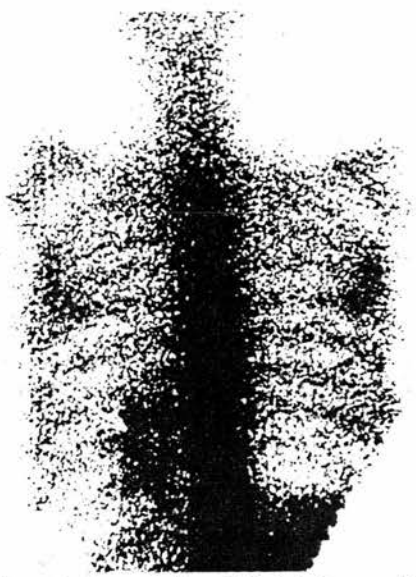

Fig 1 *Posterior image: indium-111 labelled neutrophils showing abnormal uptake (arrowed) in the left paravertebral area of the lower thorax.*

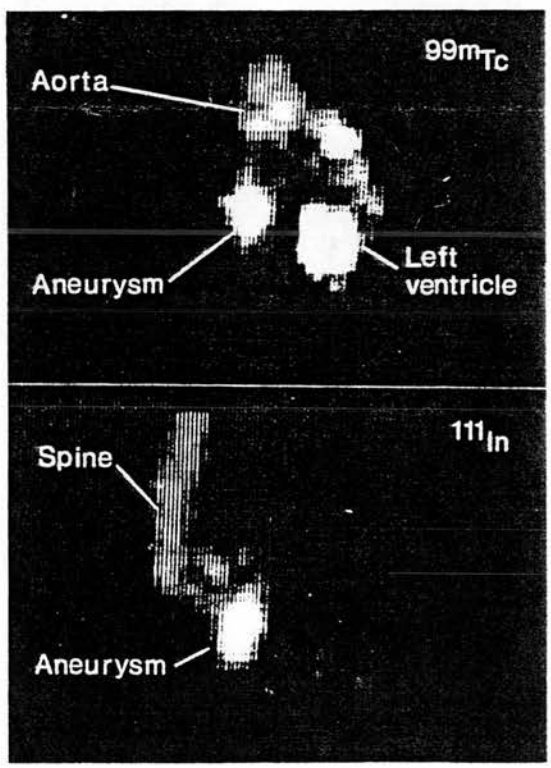

Fig 2 *Simultaneously acquired computer reconstruction images in the same sagittal plane. Upper: Technetium-99m (^{99m}Tc) labelled human serum albumin showing blood pool in the left ventricle, aorta, and region of the aneurysm. Lower: Indium-111 (^{111}In) labelled neutrophils showing abnormal uptake at the site of the aneurysm, with normal uptake in the spine.*

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showed slight irregularity of the abdominal aorta, with a patent aorto-femoral and femoro-femoral graft. Treatment with flucloxacillin was started on admission and one week later the patient underwent excision of the sinus in the left iliac fossa. The histological appearances were those of a simple "stitch sinus." Despite these measures the pyrexia persisted and further investigations to identify the cause were instituted. Repeated blood, urine, and sputum cultures failed to grow any organism. Chest radiography and computed tomography of the abdomen showed no abnormalities (photographs supplied not published). Abdominal ultrasound examination showed no abnormality. Six weeks after admission to hospital a neutrophil scan showed uptake in the left paravertebral area of the lower thorax extending from T7 to T11 (fig 1). To improve anatomical localisation, dual isotope single photon emission computed tomography was performed with technetium-99m labelled human serum albumin to allow simultaneous imaging of the blood pool. This showed uptake of ^{111}In labelled neutrophils in the same position as the descending thoracic aorta, suggesting the diagnosis of a mycotic aneurysm (fig 2). An ascending aortogram then confirmed the presence of a saccular aneurysm in the descending thoracic aorta. The patient underwent emergency surgery and a 5 cm aneurysm, adherent to the left lower lobe, was resected and replaced with a low porosity Dacron graft. The aneurysm contained clot and liquified atheroma, from which *Staphylococcus aureus* was subsequently grown. Unfortunately, the patient died five weeks after operation.

Discussion

Autologous neutrophil scanning is not frequently required or performed for the detection of occult infection in the thorax, but this case demonstrates the usefulness of whole body imaging even when an extra-abdominal source is not suspected.[4] The use of a medium energy isotope, indium, allowed further interrogation of the area of abnormal uptake in the thorax when it was combined with the low energy isotope technetium as a blood pool marker. By using single photon emission computed tomography we could infer that the area of infection lay adjacent to or within the descending thoracic aorta, a finding confirmed at aortography and surgery. We believe that this is the first case report of detection of a mycotic aneurysm of the aorta by a ^{111}In labelled autologous neutrophil scan; while other techniques, such as ultrasound or computed tomography, could have detected an aneurysm, they could not have confirmed that this was the source of infection.

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The acute inflammatory response to myocardial infarction: imaging with indium-111 labelled autologous neutrophils

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SUMMARY The uptake of indium-111 labelled neutrophils was examined in 30 patients with acute myocardial infarction by planar imaging and single photon emission computed tomography. The time from venepuncture to reinjection of the autologous labelled neutrophils was <2·5 hours and imaging was carried out 24 hours later. Twenty three patients had a positive uptake of neutrophils in the myocardium and imaging was improved by single photon emission computed tomography. There was a significant difference between the intervals from the onset of chest pain to injection of labelled neutrophils between patients with positive and negative images; early reinjection was more likely to produce a positive image. Indeed, all nine patients reinjected within 18 hours of the onset of symptoms had positive images.

The results suggest that the stimulus for activation and migration of neutrophils is transient; this is an important factor if neutrophil release products play a role in cell damage after coronary occlusion.

After myocardial infarction, myocardial cell death and damage produce an acute inflammatory response characterised by the migration of neutrophils into the area of infarcted muscle. Histological examination shows neutrophil infiltration into the infarcted area within 24 hours and the response is maximal at 4–5 days.[1 2] As part of the inflammatory response, neutrophils release oxygen derived free radicals and proteolytic enzymes that in certain circumstances may increase tissue injury. To date, the neutrophil has been implicated in damage to pulmonary capillaries in adult respiratory distress syndrome and may also contribute to the pathogenesis of emphysema. No definite role has been established for the neutrophil extending myocardial damage in man, but in animal models of myocardial infarction, infarct size can be limited by neutrophil inhibition.[3 4]

Although experimental studies have shown uptake of indium-111 (^{111}In) labelled neutrophils in infarcted myocardium[5 6] studies in man have produced conflicting results. McDougall *et al* did not detect uptake of labelled cells in three patients with acute infarction.[7] The time of injection of labelled cells may be of importance, however, because Davies and colleagues obtained positive images when the time to reinjection was short.[8] They also found that positive images were more likely in younger patients.

We have used ^{111}In labelled autologous neutrophils in 24 patients with acute myocardial infarction. Our results confirm the importance of early injection of the neutrophils in obtaining a positive image. We also found that reconstructional imaging from single emission photon computed tomography can be used to resolve difficulties in the interpretation of planar imaging.

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Patients and methods

PATIENTS

We studied 30 patients with a diagnosis of acute myocardial infarction based on a history of prolonged ischaemic chest pain (> 30 minutes), electrocardiographic changes associated with myocardial infarction, and a rise in serum creatine kinase to at least twice the upper limit of normal. All gave informed consent, and the study had the approval of

our institute's ethical committee. Table 1 shows patient details and a full record of the drugs administered within the first 24 hours of myocardial infarction.

PREPARATION OF ^{111}IN LABELLED NEUTROPHILS

Autologous neutrophils were separated from whole venous blood and labelled with ^{111}In-oxine.[9] We used an aseptic technique to withdraw whole blood (60 ml) into a syringe containing 300 units of preservative free heparin. Duplicate samples (25 ml) of blood were layered over 12 ml of mono-poly resolving medium (Flow laboratories) in a sterile tube and centrifuged at 400 *g* for 60 minutes. This produced a top plasma layer, two distinct cell bands, and a red cell pellet. We collected 8 ml from the top plasma layer and centrifuged it at 1000 *g* for 10 minutes to obtain platelet poor plasma. The remaining plasma and upper cell band were discarded and the neutrophils were recovered from the lower cell band. The neutrophils were washed by diluting the recovered cell suspension to 40 ml with phosphate buffered saline pH 7·4, centrifuging at 400 *g* for 10 minutes, and discarding the supernatant. The cell pellet was resuspended in 10 ml phosphate buffered saline and 1 ml of ^{111}In-oxine solution (20–40 MBq) was added drop by drop to the suspension of neutrophils. After incubation at room temperature for 15 minutes, 3 ml platelet poor plasma was added and the cell suspension was centrifuged at 250 *g* for 10 minutes. The supernatant was discarded, the neutrophil cell pellet was resuspended to a total volume of 5 ml with equal parts of phosphate buffered saline and platelet poor plasma and the labelled cells were then ready for reinjection.

PATIENT IMAGING

All patients were injected at a fresh site with ^{111}In-labelled autologous neutrophils within two and a half hours of the initial venesection. Preliminary studies had suggested that the optimal time for imaging was 24 hours after the injection of labelled neutrophils and we used this imaging time in all our patients. Thus the earliest imaging time for any patient was 36 hours after the onset of chest pain and the latest was 57 hours. Each patient was imaged while supine and planar images were acquired in the anterior, left anterior oblique, and left lateral position for 100 000 counts with a gamma camera (GE400 AT). In 24 patients single photon emission computed tomography was performed with the same gamma camera linked to a DEC PDP11/23+ computer that used locally written software. Ten minutes before the single photon emission computed tomography study, 40 MBq of technetium-99 m (^{99m}Tc) human serum albumin was administered to allow blood pool imaging. A sequence of 64 simultaneous images of ^{99m}Tc and ^{111}In was then acquired as the head of the gamma camera rotated through 180°, starting in the right anterior oblique position. The total imaging time was 32 minutes. At the end of this period computerised reconstruction of the images was performed.

IMAGE INTERPRETATION

An observer who was unaware of electrocardiographic findings or the maximum creatine kinase rise graded planar and single photon emission computed tomography images as positive (in which indium activity was clearly seen in the region of the heart) or negative (where there was no detectable

Table 1 *Patient details and results of imaging with indium-111 labelled autologous neutrophils*

	Positive image (n = 23)	*Negative image (n = 7)*	*Statistical significance*
Age (years)	62·0 (10·8)	59·8 (9·4)	NS (1)
Sex	(6F; 17M)	(2F; 5M)	NS (2)
Location of acute myocardial infarct	10 inf: 13 ant	3 inf: 4 ant	NS (2)
Interval from onset of chest pain to injection of ^{111}In neutrophils (h)	20·3 (6·4)	27·6 (5·8)	$p < 0·02$ (3)
Peak creatine kinase (u/l)	2023·5 (916·0)	1825 (1214·0)	NS (1)
Total leucocyte count (10^9/l)	12·9 (3·2)	12·5 (3·4)	NS (1)
Number of neutrophils injected ($\times 10^8$)	2·7 (0·9)	2·6 (0·7)	NS (1)
Activity of ^{111}In administered (MBq)	32·8 (8·4)	29·4 (8·2)	NS (1)
Drugs administered:			
Non-steroidal anti-inflammatory drugs	5	1	
Lignocaine	2	3	
Calcium antagonists	1	2	
Nitrates	7	1	

All values are shown as mean (SD).
(1) Unpaired *t* test.
(2) Exact probability test.
(3) Unpaired Wilcoxon rank sum test.

activity or where there was an area of activity on planar imaging that was inseparable from the liver, spleen, or ribs). Dual isotope single photon emission computed tomography images were considered to be positive when indium activity was seen in all three reconstruction views and corresponded with the ^{99m}Tc blood pool image.

STATISTICAL ANALYSIS

Data from the groups were compared by means of an unpaired *t* test, exact probability test, or unpaired Wilcoxon rank sum test as appropriate. Values of $p > 0{\cdot}05$ were regarded as not significant.

Results

In 23 of the 30 patients with acute myocardial infarction there was uptake of ^{111}In labelled neutrophils within the myocardium. Three patterns of cardiac uptake were seen[5]: focal myocardial uptake (12 patients), diffuse myocardial uptake (3 patients), and "doughnut pattern" (3 patients). Figure 1 shows an example of myocardial uptake. Planar images from all patients were graded as unequivocally positive or negative. In six patients in whom the planar images were considered to be negative, single photon emission computed tomography reconstruction showed localised uptake within the myocardium (fig 2). In addition, dual isotope single photon emission computed tomography reconstruction improved anatomical localisation of the infarct by confirming uptake corresponding to the cardiac blood pool (fig 3). There is a significant difference between the intervals from onset of chest pain to injection of labelled neutrophils in the groups with positive and negative images ($p < 0{\cdot}02$) (table 1). Furthermore, all patients reinjected within 18 hours had positive images whereas positive images were increasingly less common in those injected at progressively later intervals. Other features such as age, sex, peak creatine kinase, peripheral white blood cell count, dose of ^{111}In administered, and drug treatment did not influence the imaging results (table 1).

Discussion

This study confirms that ^{111}In labelled autologous neutrophils can be used to image the inflammatory response to acute myocardial infarction in man. The increased frequency of positive images in this study (77% compared with the 58% as previously described[8]) is in part related to earlier reinjection of ^{111}In labelled neutrophils after the onset of chest pain. This suggests that the stimulus for activation and migration of neutrophils to the area of myocardial damage is transient. This temporal relation may be of particular relevance because of the current interest in reducing the extent of myocardial damage after myocardial ischaemia or infarction by the use of intravenous thrombolytic treatment[10 11] or the potential for the administration of lipoxygenase or cycloxygenase inhibitors.[3]

Thrombolytic treatment can produce coronary reperfusion and hence it could improve myocardial salvage, but it may result in other events which in themselves are potentially harmful.[12] In particular, if the inflammatory response is exaggerated as a

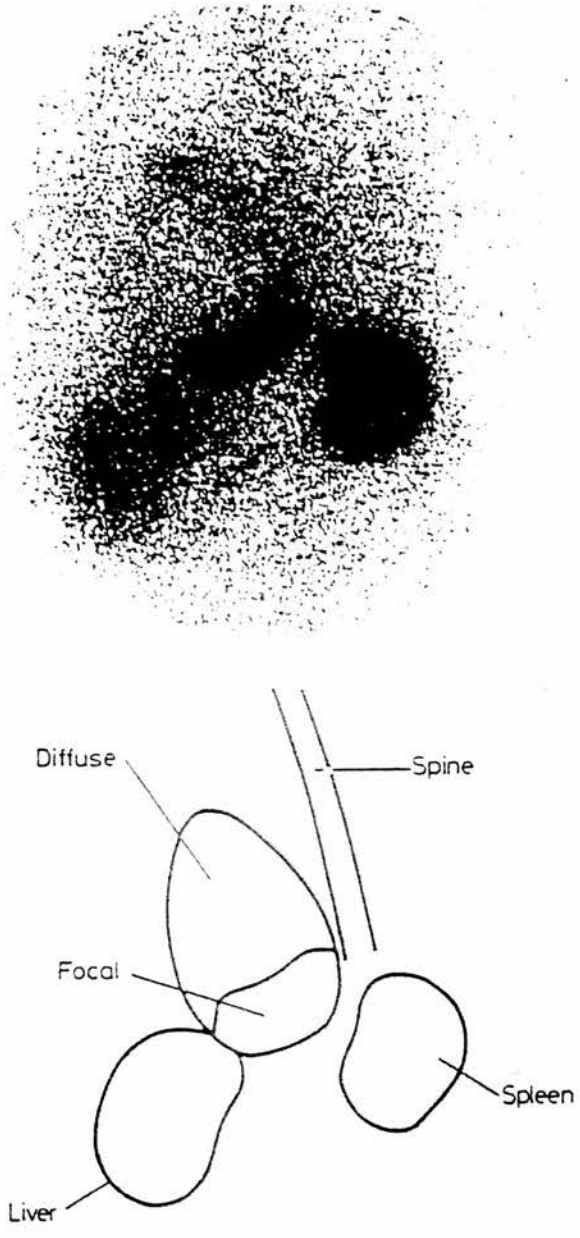

Fig 1 *Planar image in the left anterior oblique view showing normal uptake of ^{111}In labelled neutrophils in the liver and spleen and diffuse uptake in the region of the heart with an area of focal uptake in the inferior wall of the left ventricle. The line drawing shows the areas of uptake.*

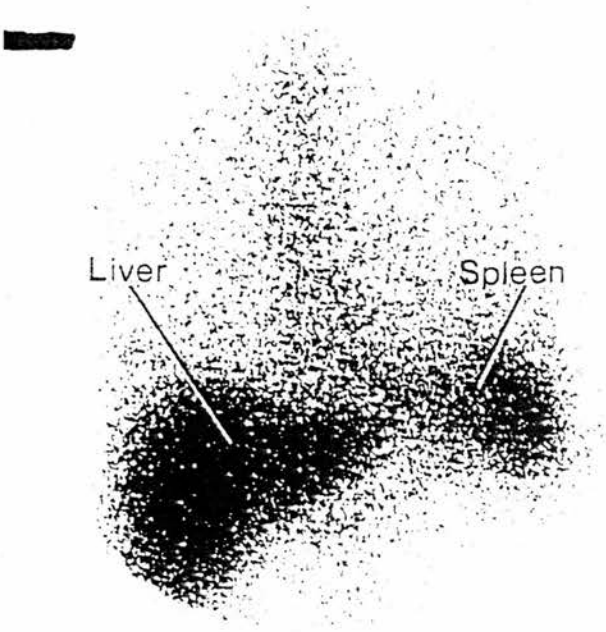

Fig 2 *Anterior planar image with normal uptake in liver and spleen and no definite myocardial uptake (top). Single photon emission computed tomographic image (bottom) in the transverse plane showing uptake within liver and spleen and an area of focal myocardial uptake (arrowed).*

result of neutrophils entering ischaemic tissue in greater numbers after reperfusion, the activated neutrophils could generate a number of cytotoxic products including oxygen derived free radicals and proteolytic enzymes, which can extend myocardial damage.[13] Both neutrophil depletion and the use of

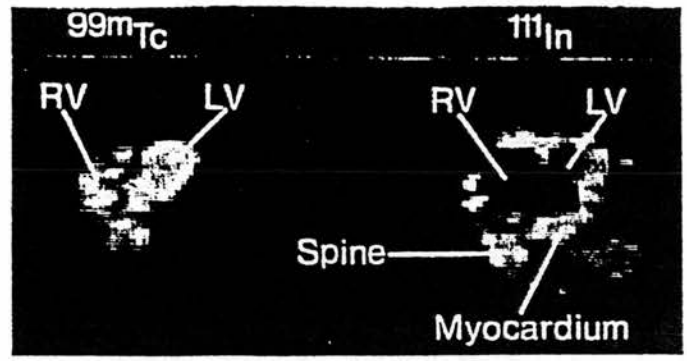

Fig 3 *Simultaneous single photon emission computed tomographic images in the transverse plane. The ^{99m}Tc image shows blood pool in the left and right ventricle. The corresponding ^{111}In image shows extensive uptake within the myocardium of both ventricles.*

non-steroidal anti-inflammatory drugs have been shown to reduce infarct size in experimental myocardial infarction,[3,4] and these findings may indicate further potential methods of improving myocardial salvage in the post-infarct period.

Single photon emission computed tomography increased the number of positive images by allowing spatial separation of positive myocardial uptake of indium from adjacent bone, liver, and spleen. It is not possible to ascertain from the planar images whether the ^{111}In activity detected in the region of the heart is due to blood pool activity. Dual isotope single photon emission computed tomography with ^{99m}Tc human serum albumin as a marker of blood pool, however, unequivocally demonstrated that the ^{111}In activity was localised in the myocardium. Single photon emission computed tomography, like pyrophosphate scans,[14] may also provide a method of quantifying neutrophil uptake within the myocardium.

Imaging with ^{111}In labelled autologous neutrophils in patients with acute myocardial infarction allows us to image the acute inflammatory response to myocardial damage, but should not be regarded as a technique for the diagnosis or localisation of acute myocardial infarction because other techniques are currently more successful. If inhibition of neutrophil migration limits the extent of myocardial

Table 2 *Imaging results*

Time (h)	*Number of patients*	*Positive images*		*Positive images %*
		Planar positive SPECT positive	*Planar negative SPECT positive*	
18	9	7	2	100
18–24	12	7	3	83
24–36	9	3	1	44

SPECT, single photon emission computed tomography.

infarction this method can be used to monitor the temporal relation and extent of neutrophil uptake in acute infarction.

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Letters to the Editor

Indium-111 Neutrophil Imaging in Ischemic Colitis

TO THE EDITOR: Indium-111 (^{111}In) autologous neutrophils are used for imaging the colon in inflammatory bowel disease (*1*) and clostridium difficile colitis (*2*) allowing noninvasive assessment of colonic involvement in these conditions but not differentiating between the different forms of colitis. We report a case where ^{111}In labeled neutrophil uptake in the colon of a patient with unsuspected ischemic colitis demonstrates the usefulness of the technique both in assessing the extent of colonic involvement and as an aid to diagnosis, but emphasise the importance of confirming the diagnosis of colitis by other techniques.

A 61-yr-old white female was referred for assessment by her family physician because of systemic hypertension resistant to therapy. Physical examination confirmed features of hypertension with elevated blood pressure at 230/115 mmHg, a grade 2/6 mid-systolic murmur at the left sternal edge and grade 2 hypertensive retinopathy.

Combination therapy with beta-blockers, diuretics, and vasodilators had failed to control the blood pressure adequately and therefore the patient was commenced on an angiotensin converting enzyme (ACE) inhibitor (captopril) and a diuretic (furosemide). This regimen caused an abrupt deterioration in renal function, blood urea rising to 22.7 mmol/l (136.2 mg/100 ml) and creatinine to 220 μmol/l (2.42 mg/100 ml). As ACE inhibition can cause reversible deterioration of renal function (*3*) in hypertensive patients with bilateral renal artery stenosis, noninvasive flow studies and aortography combined with bilateral renal angiography were undertaken and confirmed the presence of bilateral renal artery stenosis with almost total occlusion of the right renal artery. In addition, aortography demonstrated extensive atheroselerotic aneurysmal dilatation of the aorta. The patient was then referred to the Vascular Surgery Department and subsequently underwent a Dacron graft repair of the aortic aneurysm with bilateral saphenous vein grafting to the renal arteries.

Her postoperative course was complicated by the development of transient atrial fibrillation, hyponatremia, and right basal pneumonia requiring antibiotic therapy with ampicillin. On the tenth postoperative day, the patient developed profuse diarrhea, which was positive for occult blood, and the following day became septicaemic. Stool cultures at this time, yielded clostridium difficile and oral vancomycin therapy was instituted. Abdominal examination at this stage was unremarkable, but the severe diarrhea persisted and sigmoidoscopy revealed a nonspecific proctitis. To assess the degree of colonic involvement a [^{111}In]neutrophil scan was requested and ^{111}In autologous neutrophils were prepared as described elsewhere (*4*). Gamma camera images obtained 12 hr after reinjection of the cells demonstrated localized uptake in the distal and sigmoid colon with a cutoff at the level of splenic flexure in the distribution of the inferior mesenteric artery (Fig. 1). The patient's condition continued to deteriorate and she developed

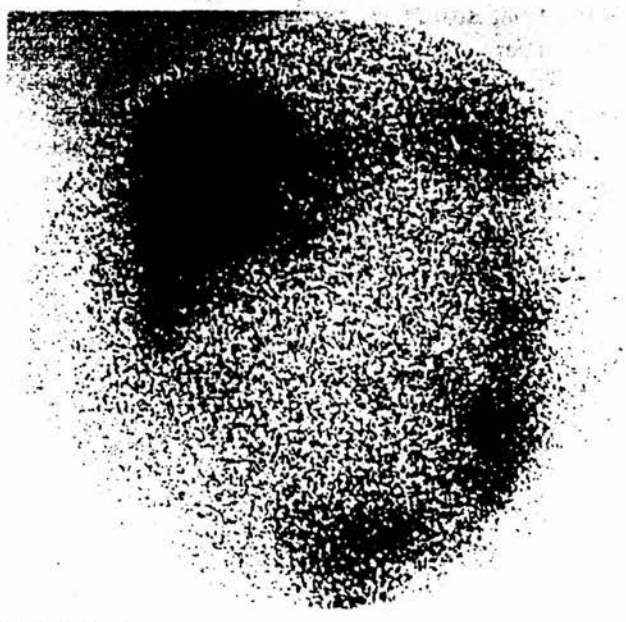

FIGURE 1
Indium-111 neutrophil image (anterior) showing abnormal uptake in sigmoid and descending colon with no uptake beyond splenic flexure

clinical and radiological signs of bowel perforation which required emergency laparotomy. At laparotomy the descending and sigmoid colon were infarcted and a left hemicolectomy and colostomy was performed. The histologic features of the resected specimen were those of an ischemic colitis, with extensive mucosal ulceration associated with a superficial inflammatory exudate. In areas there was complete loss of mucosa, submucosa and muscle with replacement by granulation tissue. There was no evidence of massive intramural hemorrhage or pericolic abscess formation. Clostridium difficile toxin assay was later reported as negative. The patient made an uneventful recovery and has had a subsequent bowel re-anastomosis. Blood pressure remains normal on no therapy.

This case illustrates the difficulty in establishing a precise diagnosis in a patient with severe diarrhea and septicaemia after major abdominal surgery. The patient might have had clostridium difficile induced colitis in view of her antibiotic exposure, elevated blood urea and recent laparotomy. This diagnosis was initially supported by the finding of clostridium difficile in the stool but the clostridium difficile toxin assay was subsequently negative. Toxin assay results are not immediately available and a positive stool culture in isolation may be misleading. The [^{111}In]neutrophil scan was useful in showing the extent of the colonic disease and more importantly, in view of the anatomical distribution, suggesting a vascular etiology. The possibility of an ischemic colitis was thus raised which prompted an immediate review of the case by the surgeons prior to the progression of her abdominal symptoms. The histology of the resected specimen demon-

strated neutrophils in the inflammatory exudate and deeper granulation tissue. In the absence of pericolic abscess formation or extensive intramural hemorrhage, the localization of ^{111}In in the descending colon demonstrates uptake of neutrophils throughout the ischemic segment of bowel. This is compatible with an inflammatory response to ischemia.

This case confirms that [^{111}In]leucocyte imaging is useful in demonstrating noninvasively the anatomical extent of colonic disease, which may also suggest alternative diagnoses such as ischemic colitis. In cases of ischemic colitis, it may provide information as to the site and extent of the disease preoperatively. This technique does not give a pathological diagnosis and it is, therefore, important to pursue this with further appropriate investigations such as colonoscopy, barium enema, or even exploratory laparotomy.

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Chylothorax on Technetium-99m Antimony Sulfide Colloid Scan

TO THE EDITOR: Interstitial injection of a radiolabeled colloid will allow visualization of regional lymph nodes. We have performed lymphoscintigraphy in two patients with chylous pleural effusions. The studies were performed with technetium-99m (^{99m}Tc) antimony sulfide colloid and satisfactorily demonstrated the abnormal thoracic localization.

The first patient, a male infant, was born prematurely at 26 wk gestation. He required surgery for necrotizing enterocolitis, and received hyperalimentation for five months. This process was complicated by bilateral subclavian vein thrombosis related to subclavian venous line placement. At the age of 9 mo he was readmitted to hospital with increasing respiratory distress. He was found to have a right-sided pleural effusion that was tapped repeatedly but which continued to reaccumulate. It was noted to be chylous in appearance, and in an attempt to better characterize the mechanism of abnormal fluid accumulation, a radionuclide scintigram was performed.

After obtaining informed consent 100 μCi of [^{99m}Tc]antimony trisulfide was injected subcutaneously into the web space between the first and second toes of each foot. Using a low-energy, all-purpose collimator, 10-min images were obtained at 2, 4, 6, and 24 hr after injection. Overlapping images allowed visualization of activity in the lower limbs, abdomen, and thorax. Lateral and oblique views helped to localize abnormal foci of activity. By 2 hr activity was noted within the right hemithorax. This was more evident by 4 hr, at which time it was mainly at the right base in the supine position (Fig. 1). The patient subsequently died from respiratory failure complicated by bilateral pneumothoraces. There were fibrous adhesions involving the distal one-fourth of the thoracic duct and the great veins at autopsy.

The second case, a 7-yr-old girl, was initially seen with staphylococcal pericarditis. Treatment at that time included stripping of the pericardium on two occasions. During surgery, the thoracic duct was damaged, and was tied off in the upper mediastinum. She then presented because of persistent coughing. Chest x-ray showed bilateral pleural effusions and prominent vascular markings. Pulmonary function tests indicated a severe restrictive defect.

Symptomatically the patient deteriorated, with persistent pleural effusion and marked engorgement of pulmonary lymphatics. She slowly became hypoxemic and was prone to

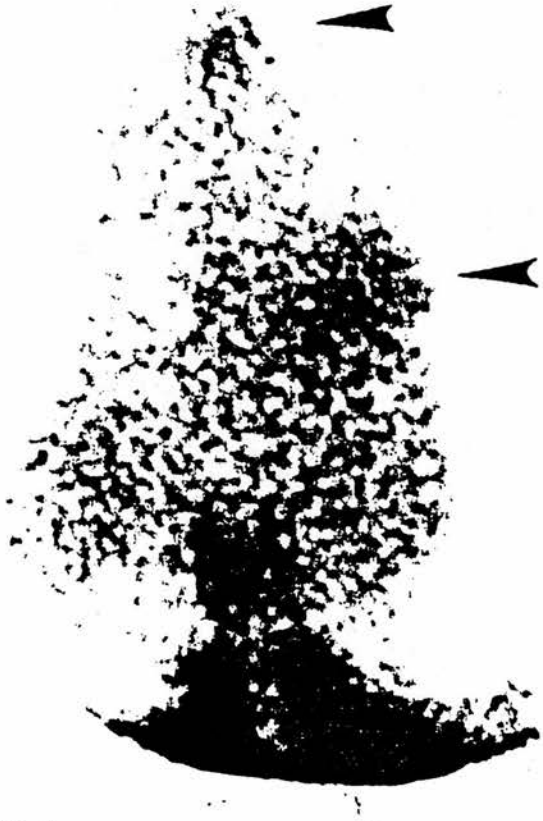

FIGURE 1
Posterior image obtained at 4 hr in 9-mo-old boy shows abnormal accumulation of activity in right lung, mainly at base (arrowheads). Activity is also noted in abdominal lymphatics and in liver and spleen

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Neutrophil activation during cell separation procedures

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Summary

Leucocytes labelled with ^{111}In or $^{99}Tc^{m}$ are used as diagnostic agents for detecting sites of infection by scintigraphy. Before radiolabelling is performed, leucocytes are isolated from whole blood. The effect of isolation procedures on neutrophil activation has been studied by measuring the neutrophil elastase produced during incubation of whole blood with three erythrocyte sedimentation agents (dextran 70, hydroxyethyl starch and methylcellulose) and two density gradient media (Percoll and Mono-Poly Resolving Medium). Neutrophil elastase was measured using a standard radioimmunoassay. At 21° C, dextran caused no elastase release while hydroxyethyl starch and methylcellulose induced significant release ($p = 0.01$ and $p < 0.01$ resp.). All three agents caused significant elastase release at 37° C. When whole blood was incubated with Percoll and Mono-Poly Resolving Medium, no release of neutrophil elastase was observed. These results show that neither density gradient medium induces neutrophil activation but that certain erythrocyte sedimentation agents do. Of the three sedimentation agents investigated, dextran is the agent of choice if neutrophil activation is to be minimized.

Introduction

Leucocytes labelled with ^{111}In or $^{99}Tc^{m}$ are used routinely in nuclear medicine as diagnostic agents for detecting sites of infection by scintigraphy. All the presently available labelling techniques require separation of the leucocytes from whole blood before labelling can be performed. The preparations of labelled cells used most commonly contain mixed leucocytes which have been obtained following treatment of whole blood with a red cell sedimentation agent [1–5]. For studies where a preparation of pure neutrophils is desired, these cells are isolated from leucocyte-rich

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plasma [6, 7] or whole blood [8] using a density gradient medium. There has been considerable interest in the methods for separating neutrophils from whole blood prior to radiolabelling because of potentially harmful effects of the separation techniques on the cells [3, 9]. Preliminary work from our laboratory suggests that it is not only the density gradient media and labelling procedures that may influence neutrophil function but also the red cell sedimentation step which is commonly employed.

Neutrophil elastase is released from 'activated' neutrophils and has been used as a marker of activation [10]. In this work we have studied the effect of cell separation procedures on neutrophil activation by measuring the concentrations of neutrophil elastase produced during incubation of whole blood with three erythrocyte sedimentation agents and two density gradient media.

Materials and methods

Effect of sedimentation agents

From each of 10 subjects, 40 ml venous blood were withdrawn into a syringe containing 200 units of preservative-free sodium heparin. Heparin was chosen since it is the anticoagulant used in our routine technique for the isolation of neutrophils [8]. A 10 ml aliquot was retained as a control sample and the remaining 30 ml was divided equally between three tubes containing a sterile solution of either 6% dextran 70 in 0.9% NaCl (4.0 ml), 6% hydroxyethyl starch in 0.9% NaCl (1.0 ml) or 2% methylcellulose in 0.9% NaCl (0.4 ml). The volume of each agent was chosen to give the concentration used routinely for erythrocyte sedimentation. The samples were gently inverted to ensure thorough mixing and then divided in two. One sample was incubated at room temperature (21° C) and the other at 37° C for 45 min. At the end of this period the supernatants were aspirated and rendered cell free by high spin centrifugation (1500 *g*) at 4° C. The concentrations of neutrophil elastase in the supernatants were assayed using a standard radioimmunoassay technique [10] and corrected to account for dilution by the sedimentation agents. Statistical analyses were performed using the Wilcoxon's test for signed ranks.

Effect of density gradient media

Whole blood (10 ml) was taken from 8 healthy volunteers and 5 ml were added to 2.4 ml aliquots of Percoll (Pharmacia) made 51% with phosphate buffered saline and Mono-Poly Resolving Medium (M-PRM, Flow Laboratories). The ratio of blood to medium was chosen to represent those used in the various methods which have been described for the isolation of neutrophils. The samples were incubated at room temperature (21° C) on a rotary mixer for 15 min to ensure continuous contact between media and blood. The samples were then centrifuged at 1500 *g* for 10 min at 4° C. The supernatants were aspirated and recentrifuged to render them cell free. Neutrophil elastase was measured as before and lactate dehydrogenase (LDH) was measured by a standard colorimetric technique to assess cell viability. The results were corrected to account for dilution by the density gradient media.

Results

The effects of the sedimentation agents are shown in Fig. 1 and demonstrate that at

room temperature, no significant difference was found between the control samples and those treated with dextran ($p > 0.05$). Methylcellulose and hydroxyethyl starch induced significant release of neutrophil elastase, compared to both the control samples ($p = 0.01$ and $p < 0.01$ resp.) and dextran ($p = 0.01$ and $p < 0.01$ resp.). At 37° C all agents were found to cause significant release of elastase.

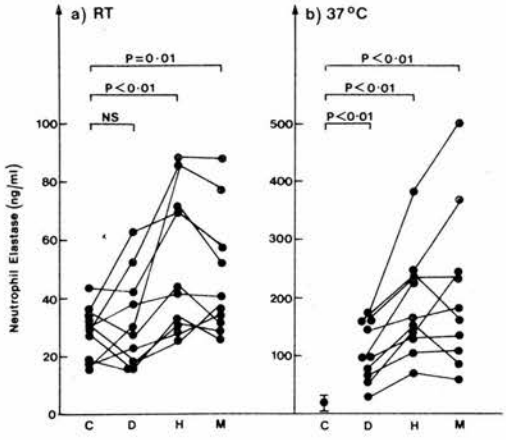

Fig. 1. Release of neutrophil elastase in 10 subjects. (a) RT = room temperature of 21° C; (b) 37° C. (C = controls; D = dextran; H = hydroxyethyl starch; M = methylcellulose).

In the experiment to investigate the effects of density gradient media, the mean results (± S.D.) for neutrophil elastase resulting from treatment with M-PRM (19.7 ± 5.7 ng ml^{-1}) and Percoll (25.1 ± 7.6 ng ml^{-1}) are within our normal range for plasma (20.8 ± 11.0 ng ml^{-1}). Similarly there was no difference in LDH between the samples (Percoll 315.6 ± 47.5 U/l, M-PRM 322.4 ± 24.2 U/l). Unlike previous studies [9, 11], these results suggest that neither M-PRM nor Percoll cause neutrophil activation.

Discussion

During the isolation of leucocytes to be radiolabelled, procedures which induce activation of the neutrophils are to be avoided. If neutrophil activation occurs, the radiolabelled cells exhibit increased adhesiveness when reinjected into the patient. The most obvious consequence of this effect is retention of labelled cells in the vasculature of the lungs, this being the first capillary bed that they encounter following intravenous injection.

Percoll and M-PRM are two density gradient media commonly used for the isolation of neutrophils to be radiolabelled. Percoll is based on colloidal silica coated with polyvinylpyrrolidone while M-PRM is a Ficoll-Hypaque mixture. It has been

suggested that, in comparison to Percoll, Ficoll-Hypaque mixtures have a detrimental effect on neutrophil function [9, 11]. In contrast to these findings, the results of our study show that neither M-PRM nor Percoll causes neutrophil activation.

This study also shows, however, that the density gradient medium is not the only step in cell separation which needs to be considered in relation to neutrophil activation and subsequent cell behaviour. Our results indicate that the red cell sedimentation agent is a more important source of neutrophil activation and that dextran is the sedimentation agent of choice if activation is to be minimized. Furthermore, the presence of neutrophil elastase, and presumably other neutrophil release products in the residual plasma following red cell sedimentation, may make this an unsuitable source of platelet-poor plasma. Moreover, as neutrophil activation is influenced by temperature, we suggest that separation techniques should be performed under controlled temperature conditions. As single step procedures for isolating neutrophils from whole blood [8, 11] do not require an initial red cell sedimentation step, we would therefore support the use of these methods to minimize possible sources of neutrophil activation.

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The preparation and *in vivo* behaviour of ^{111}In-oxine labelled neutrophils separated from whole blood using mono-poly resolving medium

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Summary

Using a single step separation procedure, we have developed a method for labelling human neutrophils with ^{111}In-oxine. This method allows a rapid separation of neutrophils from whole blood, with negligible mononuclear or red cell contamination. Preliminary studies using ^{111}In-labelled neutrophils show minimal lung retention and early accumulation in the spleen consistent with viable cells. In addition, focal accumulation of ^{111}In has been imaged in patients with localized inflammation or sepsis.

Introduction

Indium-111 (^{111}In) labelled leucocyte imaging has become a common investigation for the localization of inflammatory lesions [1–5]. The neutrophils are the predominant cell involved in the inflammatory response and it has therefore been suggested that a pure preparation of neutrophils is superior to mixed leucocytes for *in vivo* studies using ^{111}In-labelled cells [6]. The use of pure neutrophils also has the advantage of avoiding the labelling of the radiosensitive lymphocytes [7–9].

A number of methods exist to isolate pure neutrophils from blood but at present these are time consuming and involve several steps [10, 11]. Mono-poly Resolving Medium is a commercially available medium which can be used to isolate neutrophils from whole blood in a single step. In this paper we describe the isolation of neutrophils by this technique and their subsequent labelling with ^{111}In-oxine. Kinetic data and positive clinical studies are also presented.

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Methods

Patients

Investigations with ^{111}In-neutrophils were performed on 45 patients with a variety of suspected inflammatory clinical conditions. Neutrophil kinetics were studied in eight of the patients with no evidence of active lung disease.

Cell separation

Venous blood (60 ml) was collected aseptically via a 19G infusion set into a syringe containing 300 units of preservative-free heparin. A full blood count and ESR were performed on 10 ml blood. All subsequent procedures were performed using aseptic technique. In duplicate, 25 ml blood was layered over 12 ml Mono-poly Resolving Medium (Flow Laboratories) in a sterile tube and centrifuged at 400 g for 60 min. Differential migration during centrifugation results in two distinct cell bands and a red cell pellet (Fig. 1). From the top plasma layer, 8 ml was collected and centrifuged at 1000 g for 10 min to provide platelet-poor plasma (PPP). The remaining plasma and upper cell band containing mononuclear cells were discarded. The neutrophils were recovered from the lower cell band, resuspended in 40 ml phosphate buffered saline pH 7.4 (PBS) and centrifuged at 400 g for 10 min. The supernatant was discarded and the cell pellet was resuspended in 10 ml PBS. A 1 ml sample was taken to determine total and differential white cell counts and assess red cell contamination.

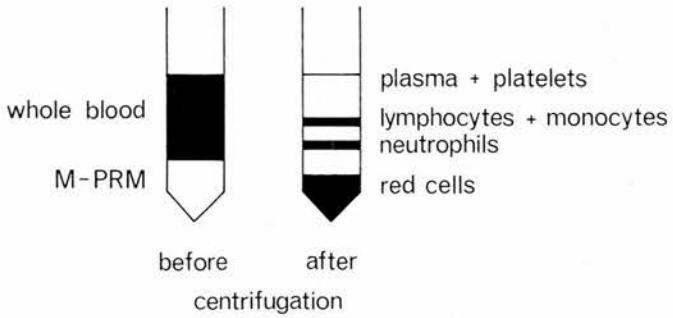


Fig. 1. Diagnostic illustration of blood cell separation on Mono-poly Resolving Medium.

Labelling procedure

^{111}In-oxine solution (20–40 MBq) (Amersham International plc) was added dropwise to the remaining suspension of neutrophils. After incubation at room temperature for 15 min, 3 ml of PPP was added then the cell suspension was centrifuged at 250 g for 10 min. The supernatant was transferred to a fresh tube. The cell pellet was resuspended to a volume of 5 ml with equal parts of PBS and PPP. The activities of the cell suspension and supernatant were measured in a radioisotope calibrator and the labelling efficiency calculated. The cell suspension which contained 18 to 40 MBq ^{111}In and 2.0 to 13.0×10^7 cells ml^{-1} was drawn into a syringe ready for injection.

Cell counting

All cell counts were performed manually using a new improved Neubauer chamber (0.100 mm). Blood films made from whole blood and the neutrophil suspension were stained with May-Grunwald/Giemsa and a differential cell count determined. The percentage cell recovery was calculated by comparing the neutrophil count in whole blood with that in the neutrophil suspension.

Scintigraphic investigations

Imaging was performed using a large field of view gamma camera (GEC-400T Maxicamera) interfaced to a PDP11-34 computer (Digital Equipment Corporation). Kinetic data were obtained from eight patients in the following manner: the patient was positioned anteriorly to visualize lungs, liver, spleen and heart and the ^{111}In-neutrophil suspension was administered intravenously by a fast running 5% dextrose infusion. A sequence of 64×64 matrix images was taken over 25 min at a varying frame interval starting at 5 s for the first six images and then increasing stepwise to 60 s for the later images. The counts were normalized for frame length. To assess ^{111}In-neutrophil kinetics, the movie images were inspected and regions of interest (ROI) created around the heart, lungs, liver, spleen and whole field of view. The whole field frame showing the maximum count-rate was assumed to represent the total activity injected. The count-rates from the other ROIs were expressed as a percentage of the maximum whole field count-rate and a time–activity curve was created for each organ. This method of data analysis allows direct comparison of the relative activity in each organ. In all patients, static images were obtained at 6 and 24 h after injection.

Results

From the 45 patients' blood samples on which separation was performed, the neutrophil recovery was 47.1% (S.D.±17.5%) and in only one sample was the

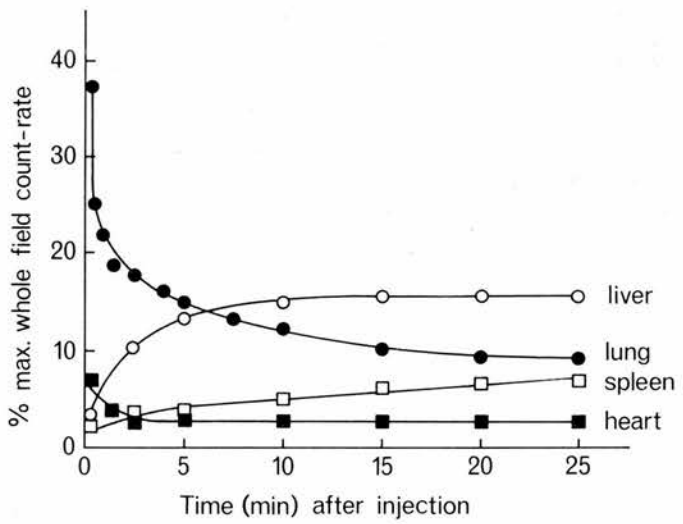
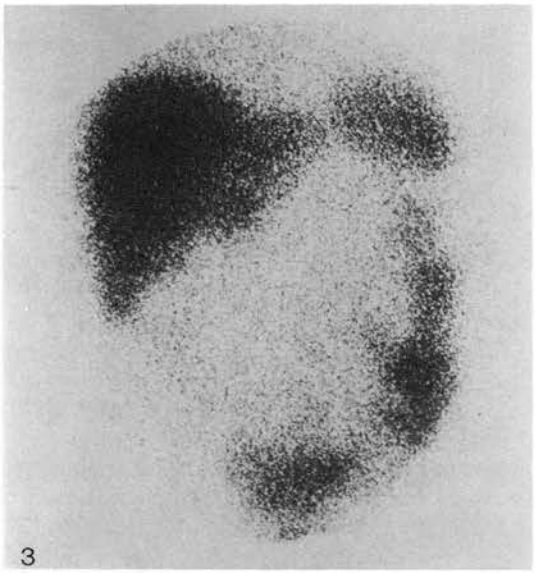
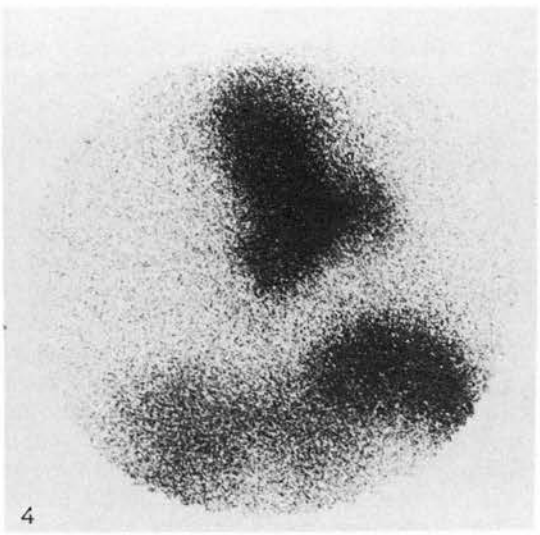


Fig. 2. ^{111}In-neutrophil time–activity curves for lungs, heart, liver and spleen.





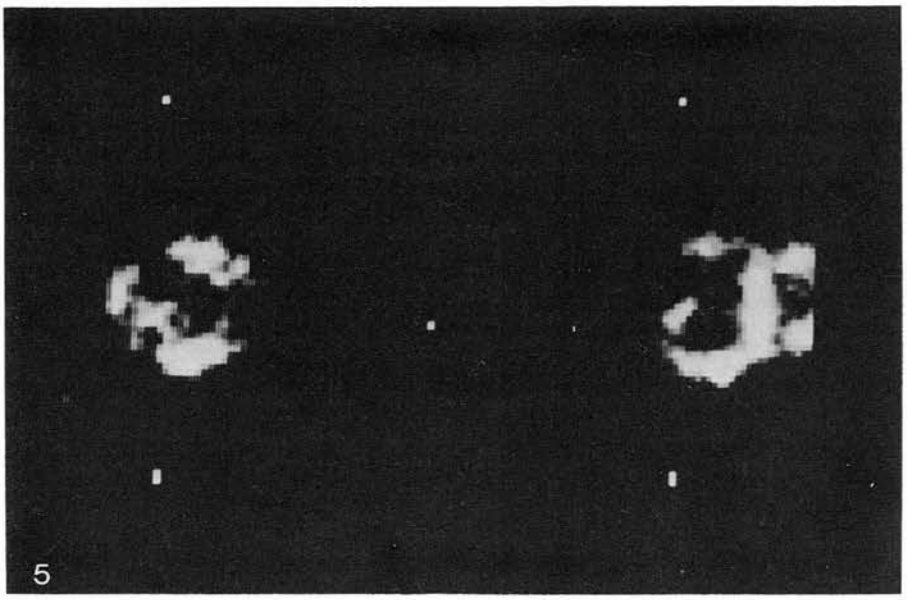


Fig. 3. Gamma camera image (24 h) showing the anterior abdominal view obtained from a patient with ischaemic colitis. Predominant uptake of ^{111}In can be seen in the sigmoid and descending colon.

Fig. 4. Anterior chest image (24 h) showing marked uptake of ^{111}In in the left upper lobe of a patient with obstructive pneumonia in the left upper lobe.

Fig. 5. Reconstructed ^{111}In transverse and coronal head images at 24 h obtained using single photon emission computerized tomography in a patient with a right frontal cerebral abscess.

neutrophil count less than 4.2×10^7. All neutrophil suspensions were labelled with ^{111}In-oxine and a mean labelling efficiency of 72.1% (S.D. ±12.9%) was achieved. In addition, red cell contamination was calculated in the eight patients used for kinetic data and was found to be 5.7% (S.D. ±2.6). Lymphocyte contamination was found to be less than 0.5%.

Fig. 2 shows the mean ^{111}In-neutrophil kinetic data obtained from the eight patients studied. The data are expressed as percentages of the maximum count rate achieved in the whole field of view. Heart count-rate falls to 3% of the maximum whole field count-rate within 90 s and after 5 min remains constant at 2% for the remainder of the study. Similarly, lung count-rate falls to 19% within the first 90 s and thereafter falls at a slower rate to 9% by 20 min indicating a small degree of lung retention. The liver count-rate rises to 16% by 15 min and remains constant, whereas the spleen count-rate continues to rise throughout the study.

Of the 45 studies, 18 showed positive uptake, three examples of which are given in Figs. 3 to 5. No patient with a negative scan was subsequently demonstrated to have a major site of infection.

Discussion

Leucocytes labelled with a variety of radionuclides have been used to locate infection or inflammation since the early 1970s [12–14]. In recent years, ^{111}In-chelates have been used to label leucocytes for routine diagnostic purposes. While much of the initial work has involved the labelling of mixed leucocytes with ^{111}In, more recently emphasis has been placed on two advantages of using pure neutrophils: (1) avoidance of labelling radiosensitive lymphocytes [7–9]. (2) Reduction in the labelling of non-specific cells principally erythrocytes and platelets [6].

A number of techniques have been developed to isolate and label pure neutrophils with ^{111}In. These methods are time consuming, requiring the preparation of a discontinuous density gradient for the cell separation. In an attempt to retain cell function it has been recommended that the neutrophils be isolated and labelled in a plasma environment. To achieve this, a technique using a discontinuous density gradient prepared with the patient's own plasma and subsequent labelling in plasma with ^{111}In-tropolonate has been developed [10]. Isolation and labelling in this manner does, however, prolong the procedure as the gradient can only be prepared after cell-free plasma has been obtained.

The purpose of this study was not to evaluate the efficacy of ^{111}In-labelled neutrophils as a diagnostic tool as this has been well documented, but to describe a technique for isolating neutrophils directly from whole blood without the need to perform an initial sedimentation step using dextran, hydroxyethyl starch or methyl cellulose as is the current practice [7, 10, 11]. Subsequent labelling of the separated pure neutrophils is achieved with ^{111}In-oxinate. This method does not require the

preparation of a discontinuous density gradient but uses the commercially available Mono-poly Resolving Medium. Good neutrophil recovery is achieved with little red cell and virtually no lymphocyte contamination. Labelling efficiency is excellent and agrees well with other published data for ^{111}In-oxinate [2, 11, 15, 16].

A number of studies has shown that poorly functioning or damaged ^{111}In-labelled neutrophils demonstrate lung retention or liver sequestration [2, 17–20]. It is essential that ^{111}In-labelled cells prepared by any new techniques of cell isolation be assessed for lung retention. One method of achieving this is to compare the passage through the lungs of a simultaneously injected ^{99}Tc^{m}-red blood cells and ^{111}In-neutrophils with simultaneous acquisition in the two energy windows [15]. Using this technique the ^{99}Tc^{m} activity–time curve represents blood flow through the lungs. Retention of ^{111}In-neutrophils is therefore demonstrated by the difference in the lung time–activity curves for the two radionuclides. We decided to use a simpler technique, comparing the ^{111}In activity–time curves for the lungs and heart, the difference in the slope of the two curves demonstrating lung retention. Kinetic data obtained from eight patients in this manner demonstrated early uptake in the liver and spleen, with splenic activity continuing to rise throughout the period of study consistent with functioning cells. Lung clearance of the ^{111}In-labelled neutrophils occurs rapidly, 80% clearing within 90 s. When the lung and heart clearance curves are compared, retention of ^{111}In in the lungs is seen, consistent with the margination of neutrophils within the pulmonary vasculature. Evidence of retention is minimal by 20 min. The difference in total lung and heart counts at 25 min can largely be explained on the basis of differences in pulmonary and cardiac blood volume and need not represent continuing lung retention of ^{111}In-labelled neutrophils. This corresponds well with the kinetic data reported in previous studies [15, 20], showing little lung hold-up with rapid pooling of neutrophils within the spleen. Further confirmation that the cells are functionally viable is provided by the positive scans obtained from a group of patients with a variety of infective and inflammatory conditions, examples of which are given earlier.

In conclusion, we have demonstrated a rapid single-step separation procedure for the isolation of pure neutrophils from whole blood using Mono-poly Resolving Medium. This method is rapid and involves less handling of cells, thus reducing the risk of mechanical damage and requiring less technical expertise. Pure neutrophils separated by this technique have good *in vivo* kinetics and appear functionally normal.

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